NeuroPhar

TARGETING THE ENDOCANNABINOID SYSTEM FOR THERAPEUTIC PURPOSES ARNAU BUSQUETS GARCIA

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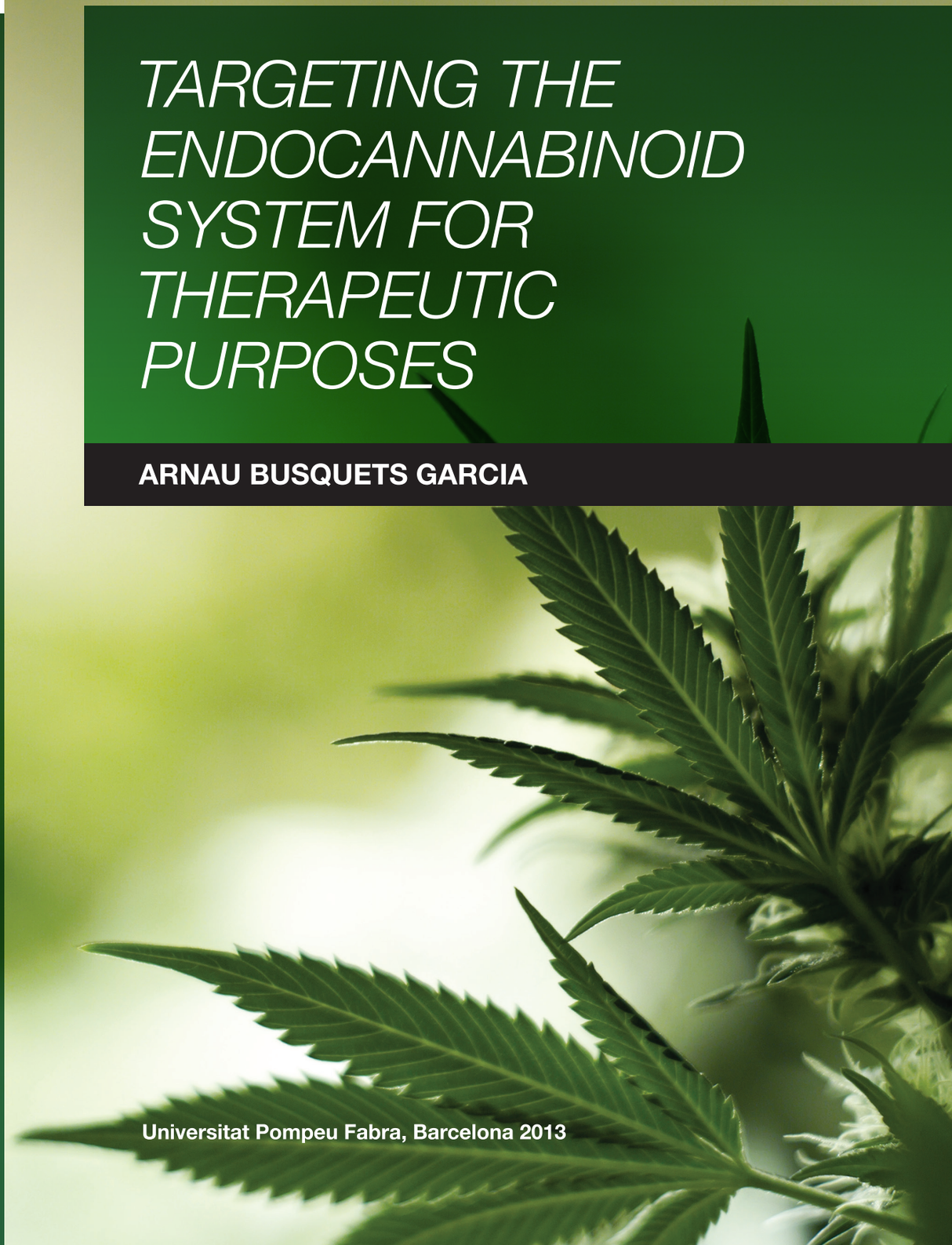

Universitat Pompeu Fabra, Barcelona 2013

TARGETING THE ENDOCANNABINOID SYSTEM FOR THERAPEUTIC PURPOSES

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A la família i a totes les persones que sempre han estat al meu costat.

“Incluso un camino sinuoso, difícil, nos puede conducir a la meta si no lo abandonamos hasta el final”

Paulo Coelho

“No hay distancia que no se pueda recorrer ni meta que no se pueda alcanzar”

Napoleón Bonaparte

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farà 10 anys que ens coneixem i poques vegades he trobat tanta complicitat i tranquil·litat per poder explicar les coses. Ets una gran persona, un crack de la ciència i com diria un amic nostre, un amic TOP. Gràcies per tot Pol!

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"I have never walked alone"

Gràcies! Gracias! Merci! Thank you!

Abstract

The endocannabinoid system is an endogenous neuromodulatory system that regulates a plethora of physiological functions, including the modulation of memory, anxiety, pain, synaptic plasticity and neuronal excitability, among others. The activation of this system through exogenous or endogenous cannabinoid agonists has been proposed as a therapeutic strategy in different pathological states, although an important caveat to their use is the possible central adverse effects, such as memory impairment, anxiety and tolerance. The activity of the endocannabinoid system has been recently found involved in the pathophysiological conditions leading to obesity and fragile X syndrome, and the blockade of this system has also been investigated as a possible therapeutic approach. This thesis mainly focuses on the behavioral, paying more attention on the cognitive effects, cellular and molecular effects of exogenous and endogenous cannabinoids in order to identify potential therapeutic effects minimizing the negative consequences associated to the cannabinoid activation. This experimental research has been centered on the modulation of the positive and negative effects of Δ9-tetrahydrocannabinol, the main psychoactive component of the *Cannabis sativa* plant, the possibility to enhance the endogenous tone of specific endocannabinoids to improve certain therapeutic applications of cannabinoids, and the effects of inhibiting the endocannabinoid system in the amelioration of different traits associated to fragile X syndrome. The combination of behavioral, cellular and molecular approaches allowed the elucidation of different important aspects of the endocannabinoid system as an interesting therapeutic target.

Resum

El sistema endocannabinoid és un sistema neuromodulador endogen que regula diferents funcions fisiològiques com la memòria, l'ansietat, el dolor i l'excitabilitat neuronal entre altres. L'activació d'aquest sistema per agonistes exògens o endògens ha estat usada com a estratègica terapèutica en diferents estats patològics tot i que els efectes adversos, com la pèrdua de memòria, l'ansietat o la tolerància, són el principal problema pel seu ús. El sistema endocannabinoid també s'ha trobat alterat en malalties com la obesitat o la síndrome del cromosoma X fràgil i, per tant, el bloqueig d'aquest sistema també s'ha emprat com a aproximació terapèutica. Aquesta tesis es centra en els efectes comportamentals i moleculars de l'administració exògena del Δ9-Tetrahydrocannabinol, el component principal de la planta *Cannabis sativa*, i en la modulació endògena del sitema endocannabinoid per tal de potenciar els efectes terapèutics minimitzant els efectes adversos dels cannabinoids. A més, en aquesta tesis també hem estudiat els posibles efectes terapèutics del bloqueig dels receptors cannabinoides en la síndrome del cromosoma X fràgil. La combinació d'aproximacions moleculars, farmacològiques, electrofisiològiques i comportamentals han permès el descobriment de diferents aspectes importants que permeten demostrar que el sistema endocannabinoid és una diana terapèutica molt interessant.

Abbreviations

2-AG: 2-arachidonoylglycerol

4E-BP: eukaryotic initiation factor 4E binding protein

Anandamide: N-arachidonoylethanolamide

MBT: marble burying test

cAMP: cyclic AMP

CB1R: CB1 cannabinoid receptors

CB1RKO: cannabinoid receptor 1 knockout

CB2R: CB2 cannabinoid receptors

CNS: central nervous system

DAG: diacylglycerol

DAGL: diacylglycerol lipase

DHPG: 3,5dihydroxyphenylglycerine

DI: discrimination index

eCB-LTD: endocannabinoid-mediated long-term depression

EMA: European Medicines Agency

EMEA: European Medicines Evaluation Agency

EPM: elevated-plus maze

ERK1/2: extracellular signal-regulated kinase 1 and 2

FAAH: fatty-acid amide hydrolase

FAAHKO: FAAH knockout

FDA: Food and Drug Administration

Fmr1: fragile X mental retardation gene

Fmr1KO: fragile X mental retardation 1 knockout

FMRP: fragile X mental retardation

GABA: γ-aminobutyric acid

KO: knockout

LDB: light/dark box

LTD: long-term depression

LTP: long-term potentiation

MAGL: monoacylglycerol lipase

MAGLKO: MAGL knockout

MAPK: mitogen-activated protein kinase

mGluR-LTD: mGluR-dependent LTD

mGluR: metabotropic glutamate receptors

mTOR: mammalian target of rapamycin

NAPE-PLD: N-acyltransferase and phospholipase D

NAPE: N-arachidonoyl-phosphatidylethanolamine

NMDA: N-methyl-D-aspartate receptor

OF: open field

p70S6K: p70 ribosomal S6 kinase

PI3K: phosphatidylinositol 3-kinase

PKA: protein kinase A

S6K: S6 kinase

THC: Δ9-Tetrahydrocannabinol

TRPV1: transient receptor potential vanilloid type 1

TSC: tuberous sclerosis complex

WT: wild-type

ZM: zero maze

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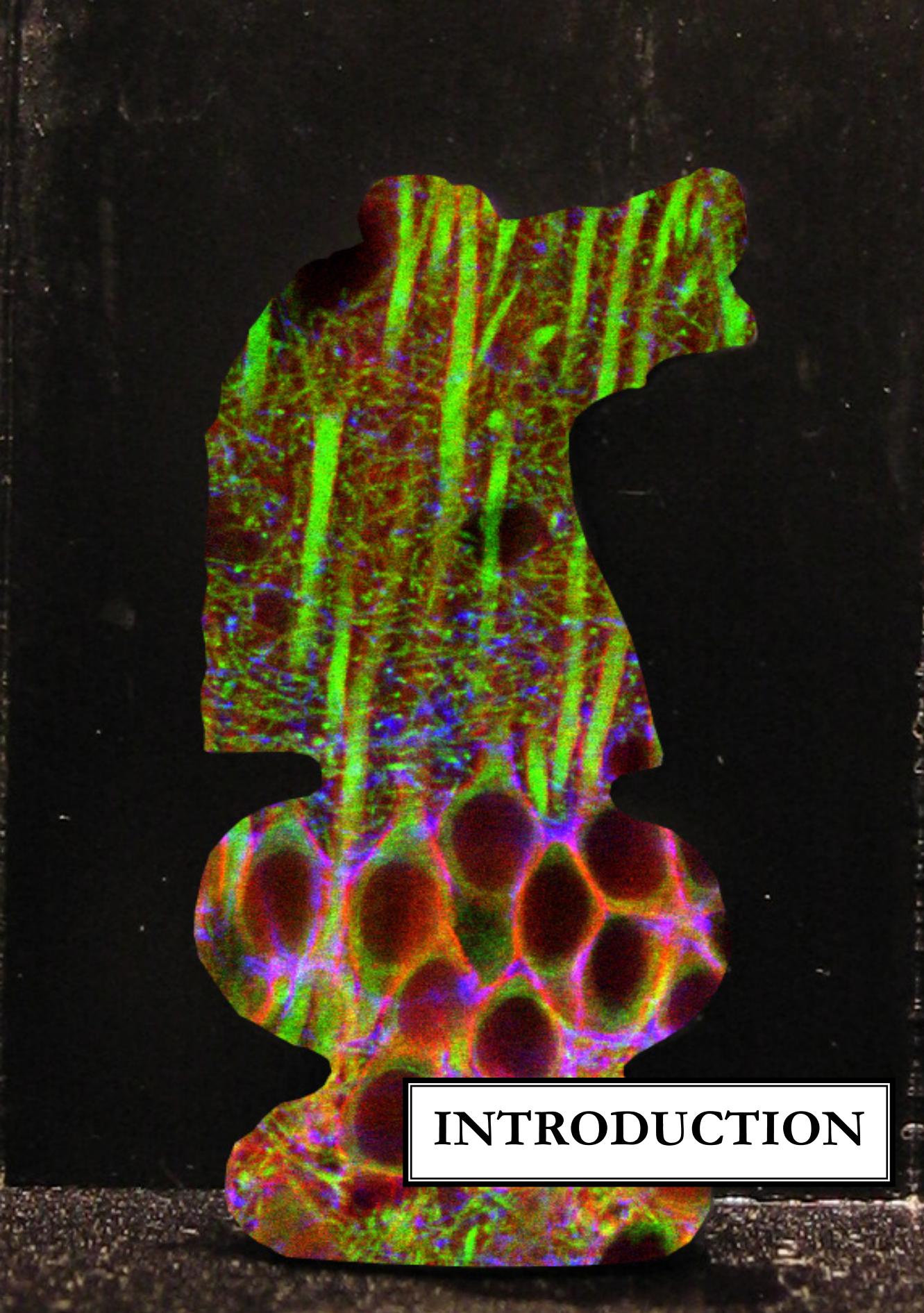


INTRODUCTION

1. The endocannabinoid system

Cannabis sativa plant has been exploited for medicinal, agricultural, recreational and spiritual purposes in different cultures over thousands of years. The use of *Cannabis* as a medicine was first recorded in China (2727 BC). This drug is considered a *pharmakon (*Greek term), which means that a substance can be a remedy with therapeutic properties as well as a poison with negative effects. During the last years, research on cannabinoid signaling has had a remarkable development.

Δ9-Tetrahydrocannabinol (THC) is the major psychoactive component of *Cannabis sativa* plant. THC structure was elucidated by Raphael Mechoulam and colleagues in the 1960s (Mechoulam and Gaoni, 1965). Since this milestone discovery, at least 70 other structurally related "phytocannabinoid" compounds have been identified. The development of synthetic cannabimimetic drugs *(Figure 1)* has aided in the pharmacological characterization of an endogenous system called endocannabinoid system.

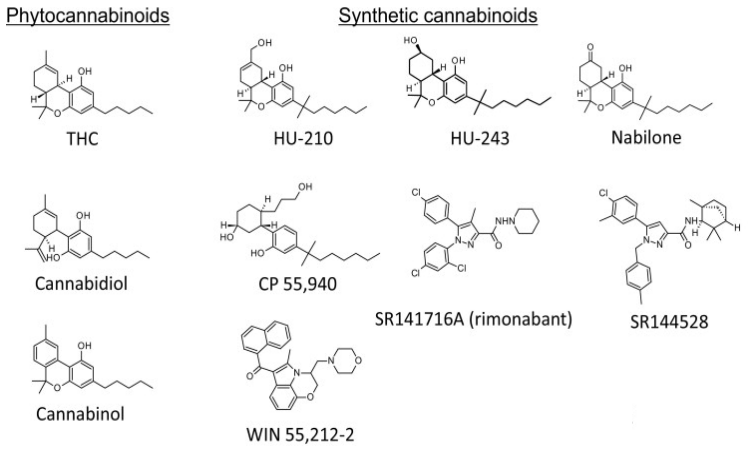


Figure 1. **Chemical structure of representative phytocannabinoids and synthetic cannabinoids** *(Maldonado et al., 2011).*

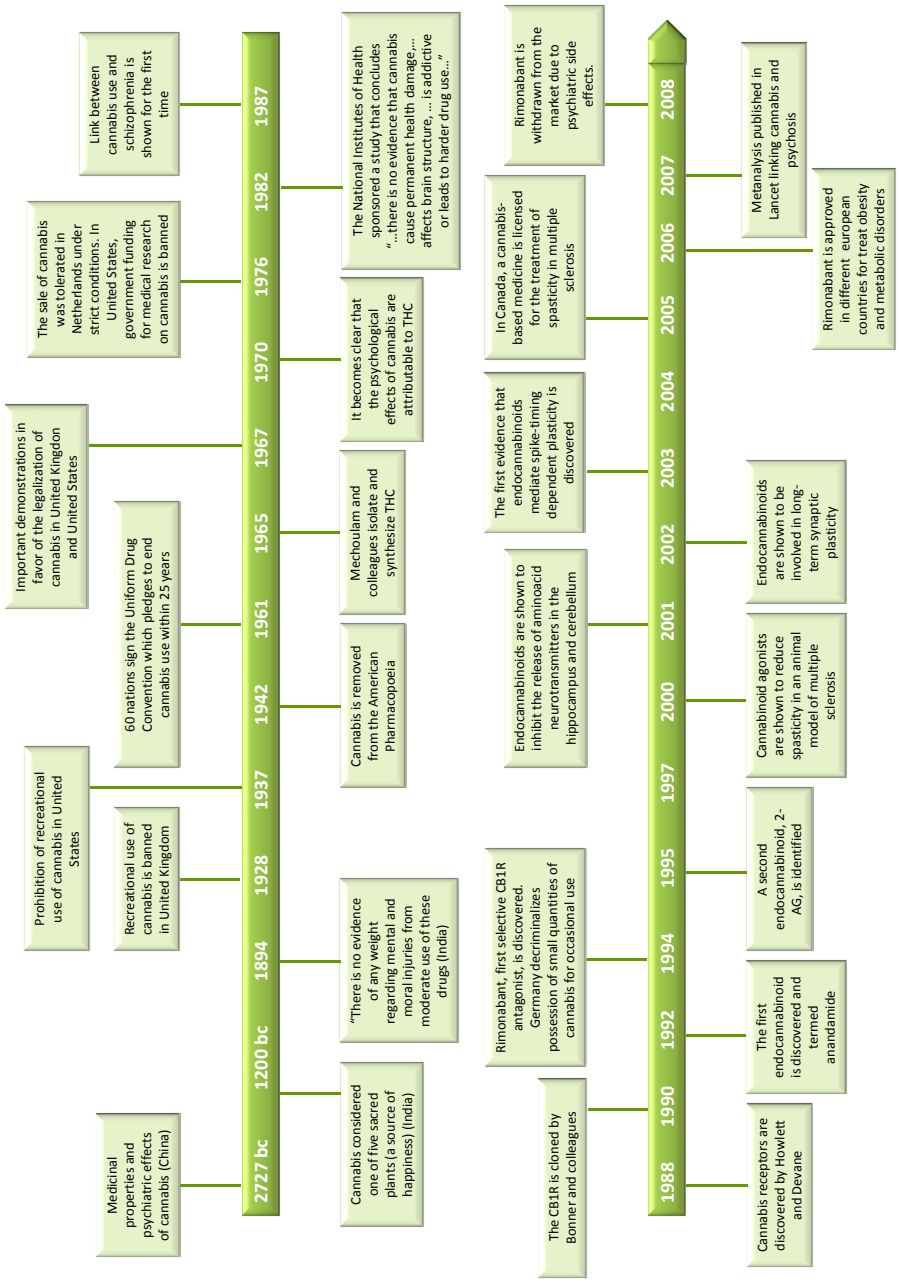


Figure 2. **Brief history of the endocannabinoid system research** *(Murray et al., 2007).*

Moreover, the serendipitous identification of a G-protein-coupled cannabinoid receptors that respond to THC (Little et al., 1988, Devane et al., 1988) gave the birth to an explosion in endocannabinoid research that continues today *(Figure 2)*.

1.1. Components of the endocannabinoid system

The endocannabinoid system is composed by the cannabinoid receptors, the endocannabinoids that are the endogenous ligands and the enzymes involved in their synthesis and inactivation. These three components will be described in the following sections.

1.1.1. Cannabinoid receptors

A major breakthrough in the cannabinoid research was the first unequivocal evidence for the presence of a specific cannabinoid receptor (Devane et al., 1988). This discovery was also considered as the first direct evidence for the existence of the endocannabinoid system.

Cannabinoids produce their pharmacological effects through the activation of at least two distinct G protein-coupled receptors: CB1 and CB2 cannabinoid receptors (CB1R and CB2R). Both receptors are members of a superfamily of seven-transmembrane-spanning. Cannabinoids differ in their affinity for cannabinoid receptors. Some of the most studied cannabinoids and their different affinity for CB1R and CB2R are summarized in *Table 1*.

Cannabinoid Receptor Ligand	K_i	
	CB_1	CB_2
	nM	
Section II.C.1		
(−)-Δ^9-THC	5.05–80.3	3.13–75.3
HU-210	0.06–0.73	0.17–0.52
CP55940	0.5–5.0	0.69–2.8
R-(+)-WIN55212	1.89–123	0.28–16.2
Anandamide	61–543	279–1940
2-AG	58.3, 472	145, 1400
Section II.C.2		
Agonists with higher CB_1 than CB_2 affinity		
ACEA	1.4, 5.29	195, >2000
Arachidonylcyclopropylamide	2.2	715
R-(+)-methanandamide	17.9–28.3	815–868
Noladin ether	21.2	>3000
Agonists with higher CB_2 than CB_1 affinity		
JWH-133	677	3.4
HU-308	>10000	22.7
JWH-015	383	13.8
AM1241	280	3.4
Section II.C.3		
Rimonabant (SR141716A)	1.8–12.3	514–13,200
AM251	7.49	2290
AM281	12	4200
LY320135	141	14,900
Taranabant	0.13, 0.27	170, 310
NESS 0327	0.00035	21
O-2050	2.5, 1.7	1.5
Section II.C.4		
SR144528	50.3–>10,000	0.28–5.6
AM630	5152	31.2
JTE-907	2370	35.9
Section II.C.5		
11-OH-Δ^8-THC	25.8	7.4
Ajulemic acid	5.7, 32.3	56.1, 170.5
Cannabinol	120–1130	96–301
Cannabigerol	81	2600
Cannabidiol	4350–>10,000	2399–>10,000
N-Arachidonoyl dopamine	250	12,000
Virodhamine	912	N.D.

Table 1. **Affinity expressed as Ki values of CB1R/CB2R ligands for the *in vitro* displacement of a tritiated compound from specific binding sites on rodent or human CB1R and CB2R** *(Pertwee et al., 2010).*

CB1R was cloned in 1990 (Matsuda et al., 1990). It is the most abundant seven-transmembrane receptor in the brain and its distribution has been well characterized both in rodents (Herkenham

et al., 1991, Tsou et al., 1998) and humans (Westlake et al., 1994). These receptors are particularly rich in certain brain areas of the central nervous system (CNS) such as basal ganglia, cerebellum and hippocampus. They are also found in other central areas including the amygdala, hypothalamus, thalamus, and the spinal cord, among other structures (Pertwee, 1997) *(Figure 3)*. CB1R are also present in the periphery, including human testis, retina, sperm cells, colonic tissues, peripheral neurons, adipocytes and other organs including human adrenal gland, heart, lung, prostate, liver, uterus, and ovary (Pertwee et al., 2010).

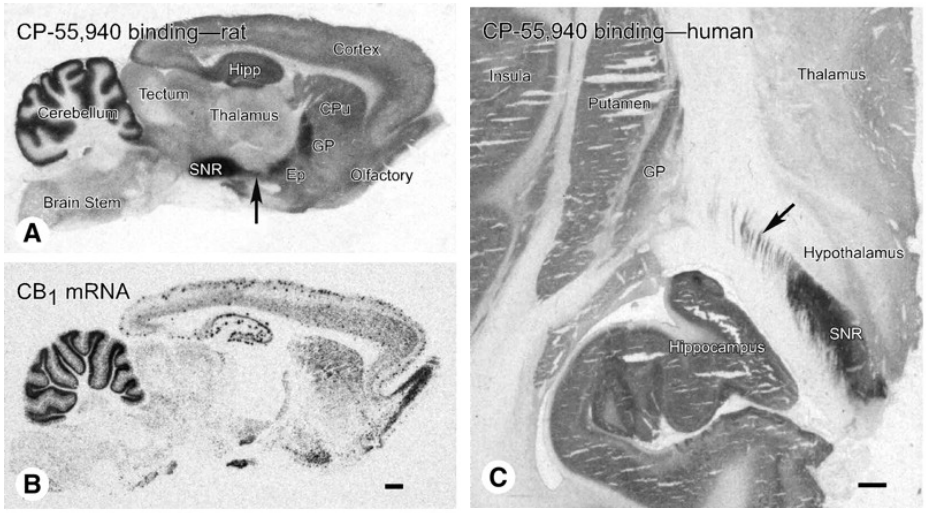


Figure 3. **Distribution of CB1R in the brain.** Autoradiographic film images (*A*–*C*) showing CB1R localization in rat (*A*) and human brain (*C*) marked by the tritiated ligand CP-55,940. Sagittal slide-mounted section of rat brain hybridized with a CB1-specific oligonucleotide probe (*B*) shows locations of neurons that express the CB1R mRNA. In both rat and human, high levels of receptor protein are visible in the basal ganglia structures. High binding is also seen in the cerebellum and in the hippocampus, cortex, and caudate putamen; and low binding is seen in the brain stem and thalamus *(Freund et al., 2003)*.

CB1R are mainly located on presynaptic terminals, where they modulate the release of a variety of neurotransmitters such as glutamate, γ-aminobutyric acid (GABA), acetylcholine, noradrenaline, dopamine, serotonin, and cholecystokinin, among others (Pertwee and Ross, 2002, Howlett, 2002, Szabo and Schlicker, 2005). Indeed, ultrastructural analysis has detected CB1R on the terminals of GABA- and glutamatergic neurons (Katona et al., 2006, Matyas et al., 2007, Eggan and Lewis, 2007). In the neocortex, the striatum and the hippocampus, CB1R expression is considerably higher on GABA- than in glutamatergic terminals (Kawamura et al., 2006). Although the reason for this remains unknown, this fact might explain the biphasic effects of THC in some phenotypes depending on the dose used. More recently, CB1R have also been localized in astrocytes (Han et al., 2012) and in a particular subcellular compartment which is the mitochondria (Benard et al., 2012).

CB2R are primarily localized to the immune system. CB2R are mainly found in the spleen, thymus and immune cells (e.g., mast cells, B- and T-lymphocytes) (Walter and Stella, 2004). The presence of CB2R in CNS is quite controversial. Although some previous studies showed that CB2R were absent in CNS neurons (Munro et al., 1993), recent studies have suggested that they exist in neurons on the brain, on dorsal root ganglia, on the lumbar spinal cord, on sensory neurons and on microglial cells (Van Sickle et al., 2005). The functional role of these central CB2R has not been clarified (Atwood and Mackie, 2010, Van Sickle et al., 2005). However, it has been shown that activation of CB2R decreases locomotion (Onaivi et al., 2006, Van Sickle et al., 2005), neuropathic and osteoarthritic pain (Guindon and Hohmann,

2008a, Jhaveri et al., 2008, Racz et al., 2008a, Racz et al., 2008b, La Porta et al., 2013). CB2R can also stimulate neural progenitor proliferation (Goncalves et al., 2008) and produce neuroprotective effects (Viscomi et al., 2009, Sagredo et al., 2009). In addition, CB2R modulate neuronal firing in the dorsal-root ganglia and the spinal cord (Elmes et al., 2004, Sagar et al., 2005), GABA-ergic transmission in the rat cerebral cortex (Morgan et al., 2009) and could also regulate cocaine's rewarding and locomotor effects (Xi et al., 2011, Aracil-Fernandez et al., 2012). These data indicate that future work to re-examine the role of functional central CB2R has to be done.

Recent studies postulate the possible existence of other cannabinoid ligand binding sites that could explain some of the physiological effects that are non-CB1R/CB2R mediated. The orphan G-protein coupled receptor GPR55 (Baker et al., 2006, Ryberg et al., 2007) has been classified as another member of the cannabinoid family. Moreover, GPR3, GPR6 and GPR12 that are sphingosine-1-phosphate lipid receptors, (Eggerickx et al., 1995, Kostenis, 2004, Uhlenbrock et al., 2003), together with the transient receptor potential vanilloid type 1 (TRPV1), are other potential cannabinoid-like receptors (Di Marzo and De Petrocellis, 2010). However, we have focused this thesis in the role of classical cannabinoid receptors, CB1R and CB2R.

1.1.2. Endocannabinoids

The discovery of the cannabinoid receptors prompted the research for their endogenous agonists. The first of these endocannabinoids to be

discovered in 1992 was arachidonoylethanolamide. It was termed anandamide from the Sanskrit word *ananda,* signifying "bliss" (Devane et al., 1992, Mechoulam et al., 1995, Sugiura et al., 1995). A second endocananbinoid, 2-arachidonoylglycerol (2-AG), was discovered in 1995 (Devane et al., 1992, Mechoulam et al., 1995, Sugiura et al., 1995), and others were soon described *(Figure 4).* Unlike conventional neurotransmitters, endocannabinoids are not stored in vesicles and are synthesized "on demand" from membrane phospholipids in response to elevations of intracellular calcium (Di Marzo et al., 2005).

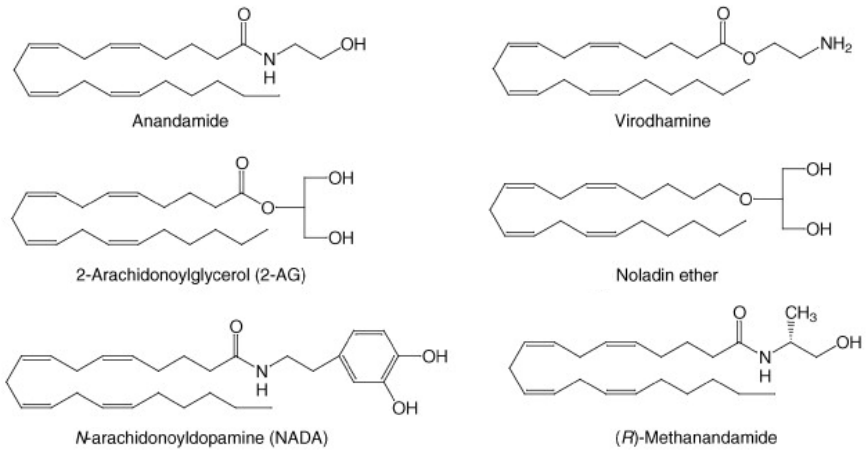


Figure 4. **Endocannabinoids structure** *(Matias and Di Marzo, 2007).*

Anandamide, as THC, acts as a partial agonist at both CB1R and CB2R *(Table 1),* and also as an endogenous ligand for the TRPV1. 2-AG, which is the most prevalent endocananbinoid in the brain, acts as a full agonist for both CB1R and CB2R. Endocannabinoids are lipids *(Figure 4)* and the balance between synthesis and inactivation finely regulates their levels. Additionally, there are other putative endocannabinoids, such as 2-arachidonoylglycerol ether (noladin ether) (Hanus et al., 2001), N-arachidonoyldopamine (Huang et al.,

2002a), and O-arachidonoylethanolamine (virodhamine) (Porter et al., 2002). However, their physiological relevance has not been indentified yet.

Endocannabinoids act as retrograde messengers at CNS synapses (Wilson and Nicoll, 2002). They are synthesized in dendrites but act presynaptically as neuromodulators preventing the presence of excessive neuronal activity and maintaining the homeostasis. Neuronal activity produces membrane depolarization. Consequently, enzymatic processes leading to the cleavage of membrane phospholipid precursors and subsequent synthesis of endocannabinoids are activated (Alger, 2002, Castillo et al., 2012, Heifets and Castillo, 2009). After endocannabinoids are released, they activate the presynaptic CB1R suppressing neurotransmitter release at both excitatory and inhibitory synapses. This inhibition could result in a short-term plasticity, as in the case of depolarization-induce suppression of inhibition or excitation (Castillo et al., 2012, Heifets and Castillo, 2009, Freund et al., 2003). On the other hand, endocannabinoids could also mediate long-term changes (Freund, Katona et al. 2003; Castillo, Younts et al. 2012) such as the endocannabinoid-mediated long-term depression (eCB-LTD). When eCB-LTD occurs at inhibitory terminals could facilitate the induction of long-term potentiation (LTP) at excitatory neurons (Chevaleyre and Castillo, 2004) *(Figure 5)*.

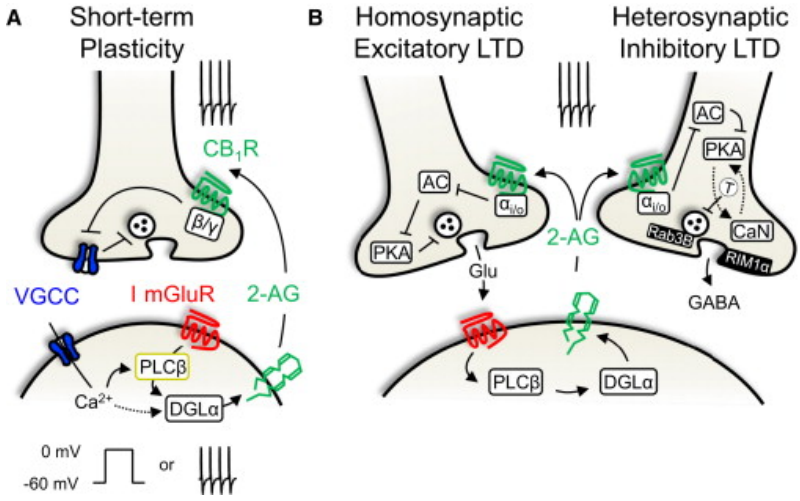


Figure 5. **Molecular mechanisms underlying endocannabinoid-mediated Short- and Long-Term Synaptic Plasticity.** (A) Short-term depression. Ca^{2+} influx and presynaptic activity lead to 2-AG production that retrogradely targets presynaptic CB1R. (B) Endocannabinoid release mediates excitatory and inhibitory long-term depression (LTD). Presynaptic stimulation releases glutamate, which activates postsynaptic mGluRs and mobilizes endocannabinoids that suppress transmitter release. At inhibitory synapses, decreased protein kinase A (PKA) activity, in conjunction with activation of the Ca^{2+}-sensitive phosphatase calcineurin, shifts the phosphorylation status of an unidentified presynaptic target required for LTD *(Castillo et al., 2012)*.

1.1.3. Enzymes involved in the biosynthesis and degradation of endocannabinoids.

Different enzymes are implicated in the synthesis and degradation of anandamide and 2-AG *(Figure 6)*. Anandamide biosynthesis mainly occurs from enzymatic cleavage of a phospholipid precursor, N-arachidonoyl-phosphatidylethanolamine (NAPE). NAPE is

synthesized by the calcium-independent N-acyl-transferase (Jin et al., 2009) and is then hydrolysed to anandamide by a specific phospholipase D (NAPE-PLD) (Di Marzo et al., 1994).

2-AG is synthesized in a two steps process. First, the 2-AG precursor diacylglycerol (DAG) is formed from enzymatic cleavage of membrane phospholipid precursors by the enzyme phospholipase C (Basavarajappa, 2007). DAG is then hydrolysed by a diacylglycerol lipase (DAGL) to generate 2-AG (Bisogno et al., 1999).

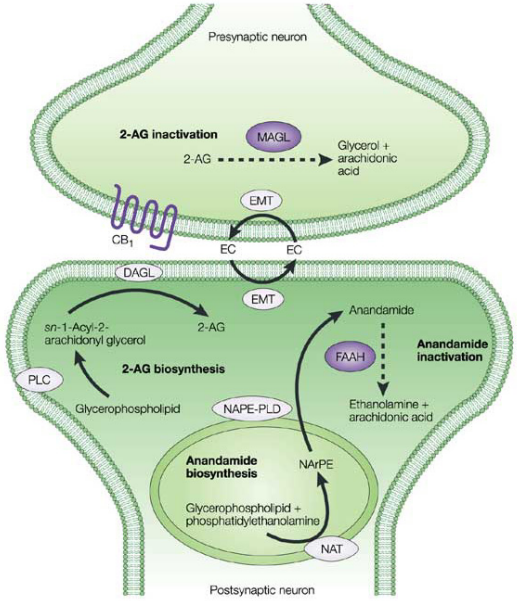


Figure 6. **Main pathways representing biosynthesis and degradation of endocannabinoids** *(Di Marzo et al., 2004).*

The enzymes that degraded endocannabinoids are quite well characterized and include fatty-acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) *(Figure 6)* (Di Marzo, 2008a, Di

Marzo, 2008b). Inhibitors for FAAH (AM374, URB597 and URB532, among others) or MAGL (URB602, OMDM169, JZL184) enzymes have been described, although the selectivity of these compounds may vary considerably. FAAH, a membrane bound enzyme, hydrolyses anandamide (Cravatt et al., 1996, Giang and Cravatt, 1997) into arachidonic acid and ethanolamine when it is present in neurons and astrocytes (Cravatt et al., 1996, Di Marzo et al., 1994). MAGL, a serine hydrolase, is the predominant enzyme that controls 2-AG hydrolysis (Dinh et al., 2002). It hydrolyses 2-AG into the breakdown products arachidonic acid and glycerol. MAGL is located on presynaptic terminals (Di Marzo, 2008a) whereas FAAH is found on post-synaptic neurons (Di Marzo 2008; Seierstad and Breitenbucher 2008). Importantly, alternative metabolic pathways for each endocannabinoid exist (Jhaveri et al., 2007, Guindon and Hohmann, 2008b). Finally, it is clear that reuptake of both 2-AG and anandamide occurs in the synaptic cleft following their release. However, the possible specific transporter proteins that mediate this uptake have not been yet identified (Hillard and Jarrahian, 2003, Alexander and Cravatt, 2006).

1.2. Cannabinoid receptor signaling

This section describes the signaling pathways that are downstream cannabinoid receptors, especially CB1R. A particular attention will be paid to the mammalian target of rapamycin (mTOR) pathway that is triggered after CB1R activation and has been involved in several THC-induced effects investigated in our studies.

1.2.1. Intracellular pathways downstream cannabinoid receptors

Stimulation of cannabinoid receptors causes a great variety of effects through the activation of numerous signal transduction pathways *(Figure 7)*. As G protein-coupled receptor superfamily members, both CB1R and CB2R mediate the biological effects by activating heterotrimeric $G_{i/o}$ type G proteins (α, β and γ). By inhibiting the adenylyl cyclase activity they reduce cyclic AMP (cAMP) and decrease PKA activity (Howlett, 2005). Moreover, CB1R coupling to $G_{\beta\gamma i/o}$ can lead to the phosphorylation and activation of multiple members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinase (Howlett, 2005). It has also been demonstrated that cannabinoids could activate protein kinase C signaling *in vitro* (Hillard and Auchampach, 1994). In addition, other proteins found to be modulated by CB1R stimulation are phosphatidylinositol 3-kinase (PI3K) (Bouaboula et al., 1995), focal adhesion kinases (Derkinderen et al., 1996), and some enzymes involved in energy metabolism (Guzman and Sanchez, 1999). The effects of cannabinoids on these multiple families of kinases indicate the relevance of changes on protein phosphorylation in the mechanism of action of these compounds. On the other hand, CB1R can also modulate various types of ion channels, including inhibition of N-type and P/Q-type calcium currents and activation of A-type potassium channels which negatively regulates neurotransmitter release (Bosier et al., 2010a). Finally, the lipid composition of the cellular membrane surrounding the receptor (Maccarrone, 2010) and the possible formation of

heteromers with other G protein-coupled receptors (Pertwee et al., 2010) seem to be critical for the regulation of signal transduction pathways triggered by G protein-coupled receptors like CB1R.

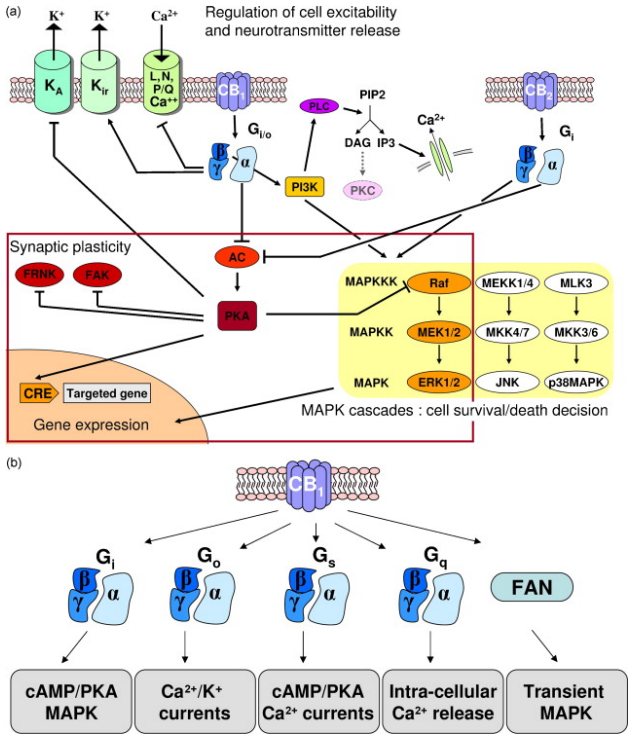


Figure 7. ***Complexity of cannabinoid receptor signaling.*** Both CB1R and CB2R are associated with $G\alpha_{i/o}$-dependent inhibition of adenylyl cyclase activity and $G\beta\gamma$-dependent activation of the different MAPK cascades (A). CB1R negatively regulate voltage-gated Ca^{2+} channels and positively regulates inwardly rectifying K^+ channels, thereby inhibiting neurotransmitter release. Cross-talk between signaling pathways are illustrated by the variety of responses requiring cannabinoid-mediated inhibition of PKA. Besides, it is now demonstrated that activation of CB1R also leads to activation of G_s and G_q proteins (B). Preferential activation of different intracellular effectors by each G protein contributes to diversity and selectivity of responses regulated by cannabinoid receptors *(Bosier et al., 2010b).*

1.2.2. mTOR pathway

mTOR is a serine/threonine kinase that acts as a downstream mediator of the PI3K/Akt pathway *(Figure 8)*. The development of selective mTOR inhibitors, such as rapamycin or its derivate temsirolimus, together with genetic approaches have allowed to demonstrate that this signaling pathway is involved in different important functions in the CNS.

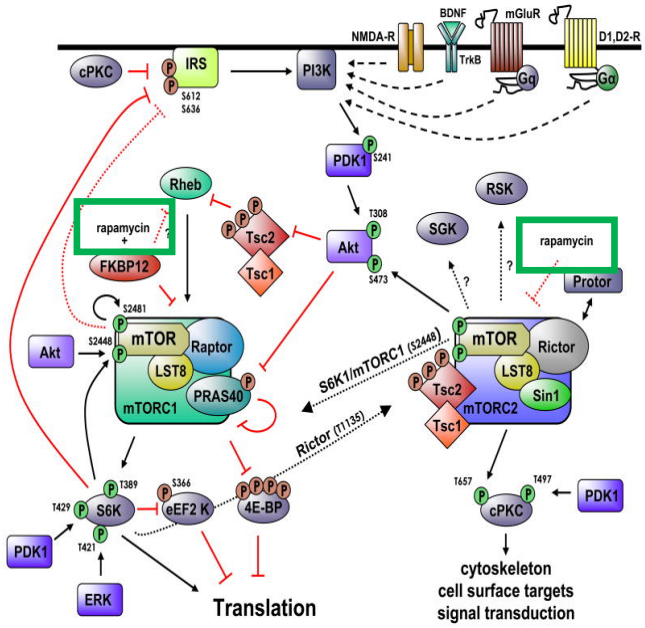


Figure 8. **Signaling upstream and downstream of mTOR, and sites of action of rapamycin.** Neuronal receptors and channels such as N-methyl-D-aspartate receptors (NMDAR), metabotropic glutamate receptors (mGluR) and dopamine receptors could activate mTOR pathway through PI3K/Akt activation. mTORC1 activity regulates several downstream effectors of translation. mTORC2 may modulate the activity of mTORC1 and other targets controlling the cytoskeleton formation. The green squares indicate the sites where rapamycin acts in the mTOR pathway *(Hoeffer and Klann, 2010)*.

Importantly, mTOR could modulate cell growth and proliferation, mRNA translation, cellular differentiation and tumor progression (Myskiw et al., 2008). This pathway is also implicated in protein synthesis, synaptic plasticity and memory processes (Hoeffer and Klann, 2010, Costa-Mattioli et al., 2009, Ma and Blenis, 2009). Moreover, mTOR has been found altered in some neuronal disorders, such as tuberous sclerosis or Alzheimer's disease (Hoeffer and Klann, 2010).

1.2.2.1. mTOR and protein synthesis

Energetically, translation is an costly process divided into three steps: initiation, elongation, and termination. An alteration in the translation process might give rise to a wide number of pathological states, such as cancer, tissue hypertrophy and neurodegeneration. The best-characterized function of mTOR is the regulation of two important components: p70 ribosomal S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein (4E-BP) (Sengupta et al., 2010). These proteins participate in the initiation process that is considered the major rate-limiting step of protein synthesis (Klann and Dever, 2004, Banko and Klann, 2008). On the other hand, mTOR also regulates several phosphatases, such as the protein phosphatase 2, which likewise controls the mTOR effectors modulating translation rates *(Figure 9)*. mTOR function can be influenced by the activities of neuronal surface receptors and channels including NMDAR, cannabinoid receptors and mGluR (Hoeffer and Klann, 2010, Puighermanal et al., 2009). The crucial role of mTOR modulating

protein synthesis prompted this pathway as a key regulator of synaptic plasticity and memory processes.

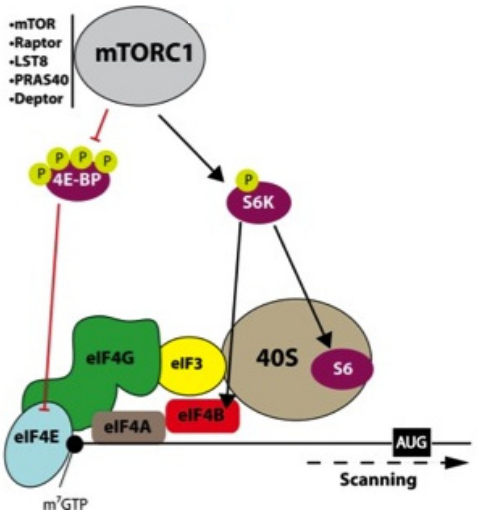


Figure 9. **mTOR signaling pathway.** Different signals could activate the mTOR complex. It phosphorylates 4E-BPs, S6 kinase (S6K), and PRAS40. Moreover, cap-dependent translation through formation of the eIF4F complex can be also regulated by mTOR inputs through 4E-BPs and S6Ks. So mTOR is a crucial regulator of translation and protein synthesis *(Gkogkas et al., 2010).*

1.2.2.2. mTOR, synaptic plasticity and memory

Synaptic plasticity is the ability of the synapse to change the strength in response to the use or disuse of transmission over synaptic pathways (Hughes, 1958). Importantly, synaptic plasticity can be also defined as temporally, when alterations last for only some seconds, or long-lasting, when synaptic changes persist over the lifetime of the organism (Kandel, 2001). In vertebrates, long-term changes in synaptic

strength are often measured as LTP and LTD. The more durable forms of synaptic plasticity need production of new proteins, both somatically and dendritically, a process where mTOR is crucially involved (Kandel, 2001, Hoeffer and Klann, 2010).

The first evidences to link mTOR signaling to synaptic plasticity were found using rapamycin in aplysia and crayfish (Beaumont et al., 2001, Yanow et al., 1998). These findings highlighted that mTOR is crucial to multiple phases of long-lasting plastic changes. Moreover, the use of rapamycin demonstrated the role of mTOR in a late phase of NMDAR-dependent LTP (Tang and Schuman, 2002). Besides, mTOR pathway, within a specific time window, regulates several components of the translational machinery, such as eIF4E and 4E-BPs, that are crucial for synaptic plasticity (Cammalleri et al., 2003, Tsokas et al., 2005).

mTOR is also important for the establishment of another form of protein synthesis-dependent synaptic plasticity, the mGluR-dependent-LTD (mGluR-LTD) (Huber et al., 2001). Indeed, mTOR signaling is activated following exposure to 3,5-dihydroxyphenylglycerine (DHPG), a mGluR1/5 agonist that induces mGluR-LTD (Hou and Klann, 2004). Furthermore, pharmacological blockade of an upstream protein of mTOR (PI3K) or the direct inhibition of mTOR by rapamycin attenuates the mGluR-LTD by reducing the phosphorylation of eukaryotic initiation factor eIF4E and 4E-BP (Banko et al., 2004, Banko et al., 2006). Interestingly, this mGluR-LTD is altered in several neuronal disorders, including fragile X syndrome (Huber et al., 2002).

Although genetic knockout mice for mTOR have not been generated in either invertebrate or vertebrate systems, the relevance of mTOR signaling in synaptic plasticity has been revealed with the recent development of novel genetic mouse models. In fact, deletion of several mTOR pathway elements including mTOR, Raptor and Rictor is developmentally lethal (Guertin et al., 2006). Perhaps this is not surprising given the crucial role of mTOR in such number of important functions for the organism. However, numerous mutant and transgenic lines for different proteins upstream and downstream of mTOR have been used *(Table 2)*.

	FKBP-12 KO	TSC1+/-	TSC2+/-	4E-BP2 KO	S6K1 KO	S6K2 KO
Morris Water Maze	=	↓	↓ ↑	↓	=	=
Contextual fear conditioning	↑	↓	↓	↓	↓	=
Early-LTP	=	*ND*	↓ ↑	↑	↓	=
Late-LTP	↑	*ND*	*ND*	↓	=	=
LTD	*ND*	*ND*	↓	↑	↑	↑

Table 2. **Long-lasting plasticity and memory phenotypes from mice with mutations in proteins upstream and downstream mTOR.** Normal (=), enhanced (↑), or impaired (↓) long-lasting synaptic plasticity and/or memory is compared with wild-type (WT) mice. ND, no data; TSC, Tuberous Sclerosis Complex *(Gkogkas et al., 2010)*.

Tsc1/2 is the farthest upstream effector of mTOR that has been genetically modified in mice. Deletion of Tsc1 has been related in

hippocampus-dependent learning and memory deficits, as well as in social behavior deficits (Bayascas et al., 2005). On the other hand, genetic ablation or reduction of Tsc2 has been involved in several synaptic phenotypes (Goorden et al., 2007), such as exaggerated LTP that can be reversed by rapamycin (Ehninger et al., 2008). Interestingly, similar phenotypes have been observed in mutations of other molecules that should increase rates of protein synthesis (Costa-Mattioli et al., 2009). Another important protein is the binding partner of rapamycin FKBP12. Conditional knockouts of this protein showed some repetitive behaviors and enhanced LTP (Hoeffer et al., 2008). Finally, mouse mutants are also available for the two most prominent mTOR substrates, S6K and 4E-BP2 (Banko et al., 2005, Antion et al., 2008a). S6KKO mice display memory impairments demonstrating that S6K function is required for proper memory formation and synaptic plasticity (Antion et al., 2008a). In addition, 4E-BP2KO mice display multiple abnormalities, including spatial learning and memory deficits (Banko et al., 2007).

1.2.2.3. mTOR alterations and neuronal disorders

The crucial function of mTOR in the regulation of protein synthesis and synaptic plasticity highlights a potential link between mTOR and several human neurological disorders (Troca-Marin et al., 2012) *(Figure 10)*. In fact, pharmacological studies and genetic mouse models have demonstrated that a deregulation of mTOR activity is found in several diseases of the CNS. Thus, an aberrant mTOR signaling has been involved in neurofibromatosis type 1, tuberous sclerosis, Down syndrome, Rhett's syndrome and others (Rosner et al., 2008, Kwiatkowski, 2003, Krab et al., 2008, Troca-Marin et al., 2012).

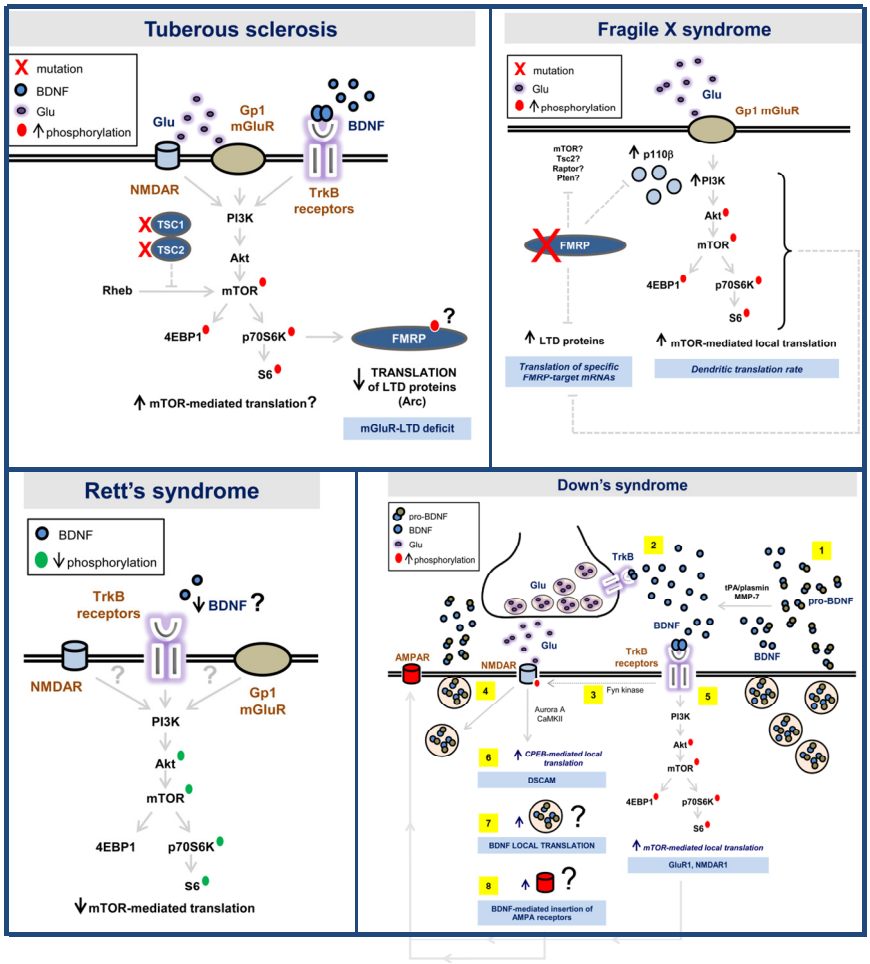


Figure 10. **Schematic representations of the mTOR alteration in different neurological disorders** A deregulated translation was found in tuberous sclerosis due to an mTOR hyperphosphorylation that increases the levels of the phosphorylated 4EBP1 and p70S6K, increasing the translation of mRNAs. On the other hand, there is a deregulation of local translation in fragile X syndrome as a result of an mTOR over activation. In the Rett's syndrome the hiperphosphorylation of different mTOR components results in lower rates of translation. Finally, in Down syndrome, the hyperactivation of the mTOR pathway induces an increased postsynaptic excitatory activity, contributing to the excitatory loop of this disease *(Troca-Marin et al., 2012).*

On the other hand, components of the mTOR pathway have been recently related to some autism spectrum disorders (Hoeffer and Klann, 2010). In this regard, fragile X syndrome, the leading genetic cause of autism, shows a mTOR signaling deregulation that has been proposed as one of the possible causes of this disease (Sharma et al., 2010a). Finally, a growing body of evidences suggests that mTOR plays a critical role in neurodegenerative diseases modulating autophagy, such as Alzheimer's disease or Huntington's disease (Rami, 2009, Swiech et al., 2008, Ravikumar et al., 2004). These numerous links between mTOR and neuronal disorders suggest that mTOR inhibitors may have significant therapeutic potential in the next future.

1.3. Role of the endocannabinoid system.

It is known that the endocannabinoid system plays a crucial role in different functions in the CNS *(Figure 11)*. Innovative approaches combining different genetic and pharmacological tools have been used to examine specific endocannabinoid system functions that will be discussed in this section.

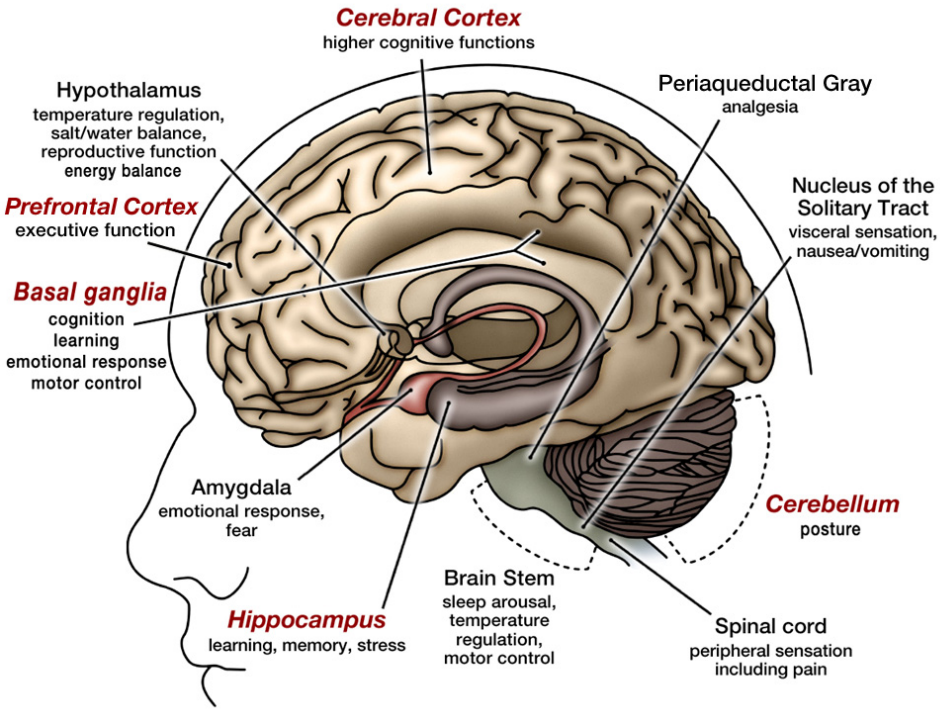


Figure 11. **Main physiological functions of the endocannabinoid system**. The brain regions where it is found higher expression of the CB1R are marked in red. The different brain regions where the endocannabinoid system has been located modulate different physiological functions in the CNS.

1.3.1. Main functions of the endocannabinoid system

The widespread distribution of the endocannabinoid system in the CNS correlates with its role as a modulator of multiple physiological functions *(Figure 11, Table 3)*. Extensive research in the last two decades has consolidated our view on endocannabinoids as powerful regulators of synaptic function throughout the CNS. As a retrograde system, the endocannabinoid system could regulate neurotransmitter release both in excitatory and inhibitory synapses (Katona and Freund, 2012, Kano et al., 2009), synapse formation and neurogenesis (Harkany et al., 2008), synaptic remodeling (Derkinderen et al., 1996, Kano et al., 2009), neuronal differentiation (Rueda et al., 2002) and neuronal survival (Panikashvili et al., 2001, Marsicano et al., 2003). Overall, the endocannabinoid system is a major homeostatic mechanism to maintain the structure and function of the brain circuits.

	Physiological role	Pathological situations
Central nervous system	Synaptic plasticity (LTP, LTD) Neuronal excitability Reward/motivation Emotions Learning/memory Locomotion Appetite Supraspinal nociception	Ischemia, anxiety/neuropsychiatric disorders, depression, memory loss, excitotoxicity, epilepsy/spasticity, drug addiction, Alzheimer's disease, Post-traumatic stress disorders, Parkinson's disease, hyperplasia/obesity, chronic pain
HPA axis	Stress response	Chronic stress
Sensory nervous system	Nociception/inflammation	Neuropathic/inflammatory pain
Immune system	Cytokine release Chemotaxis Leukocyte differentiation Inflammation response	Autoimmune disorders/atherogenic inflammation, allergy
Cardiovascular system	Vasodilatation/blood pressure Cardiomyocyte protection	Hypertension/hypotension, ischemic damage
Respiratory system	Airway smooth muscle relaxation	Cough and bronchial hyper-reactivity
Gastrointestinal system	Motility and secretion Inflammation	Nausea, satiety, reflux, diarrhea, paralytic ileus, inflammatory bowel disorders.
Liver	Lipogenesis Hepatocyte/cell function	Fibrosis, triglyceride metabolism
Adipose organ	Lipogenesis	Adipocyte hypertrophy, abdominal obesity
Reproductive system	Embryo implantation Sperm motility	Pre-term abortation, extra-uterine pregnancy, male infertility
Bone	Bone formation	Osteoporosis, ectopic bone formation

Table 3. **Physiological and pathological situations where the endocannabinoid system plays a critical role.** The endocannabinoid system is localized in multiple central and peripheral tissues. This system plays a large variety of physiological roles (second column) and participates in different pathological states (third column).

At the central level, CB1R are located at different brain structures that control distinct physiological functions *(Figure 11, Table 3)*. In the olfactory system, CB1R are responsible for the modulation of olfaction in humans and rodents (Egertova and Elphick, 2000). Moreover, the presence of CB1R in the basal ganglia and cerebellum has been related to the fine control of movement and motor coordination (Rodriguez de Fonseca et al., 1998). It is also a key modulator of emotions, motivation (Mechoulam and Parker, 2013), reward functions controlling food intake and drug addiction (Bellocchio et al., 2010, Maldonado et al., 2011) and pain (Guindon and Hohmann, 2009), among other physiological roles. On the other hand, the hippocampus has been investigated more extensively than other brain regions due to the clear deleterious effects of cannabis on learning and memory (Kano et al., 2009). Interestingly, the endocannabinoid system is also present at the peripheral level where it modulates the immune system, vascular beds, reproductive organs, gastrointestinal motility and metabolism, among others (Grotenhermen and Muller-Vahl, 2003).

In this section we will describe more in detail the specific role of the endocannabinoid system in anxiety, pain and neuronal excitability because these are the specific functions studied in this thesis. In addition, a complete section (Section 2) is devoted to the involvement of the endocannabinoid system in cognition because this is the main focus of our research activities included in this work.

1.3.2. Anxiety

Anxiety, which is a normal human reaction to a variety of stressful conditions, can be regulated by a variety of endogenous neurotransmitters. Considerable data exist on the direct effects of exogenous and endogenous cannabinoids on anxiety in animals (Viveros et al., 2005). Numerous studies *(Table 4)* have investigated the effects of cannabinoid agonists in emotional responses although contradictory results have emerged due to the dosage, genetic background and environmental context (Moreira et al., 2009, Saito et al., 2010). It is known that cannabinoid agonists display biphasic effects, eliciting anxiolytic-like responses at low doses, whereas higher doses induce anxiogenic-like effects (Moreira et al., 2009). However, more research is needed to understand the mechanisms of these bimodal effects.

The physiological role of the endocannabinoid system in the regulation of anxiety has been studied in animals using specific inhibitors of the enzymes that hydrolyze endocannabinoids *(Table 4)*. Potent, selective and systemically active carbamate-based inhibitors have been developed to inhibit FAAH, the enzyme responsible for the degradation of anandamide (Kathuria et al., 2003). The best inhibitors in these series (URB532 and URB597) exert anxiolytic properties in different anxiety tests in different animal models (Moreira et al., 2008, Kathuria et al., 2003). Moreover, experiments using FAAH knockout (FAAHKO) mice confirmed that anandamide has anxiolytic-like properties through the activation of CB1R (Cippitelli et al., 2008). In this regard, it is important to mention that anandamide is released in response to anxiogenic situations in the amygdala (Gaetani et al., 2003,

Marsicano et al., 2002). On the other hand, the enhancement of 2-AG levels by inhibiting the main enzyme involved in 2-AG degradation, MAGL, produces similar effects by reducing the anxiety-like behavior in different paradigms (Sciolino et al., 2011, Mechoulam and Parker, 2013) *(Figure 12)*.

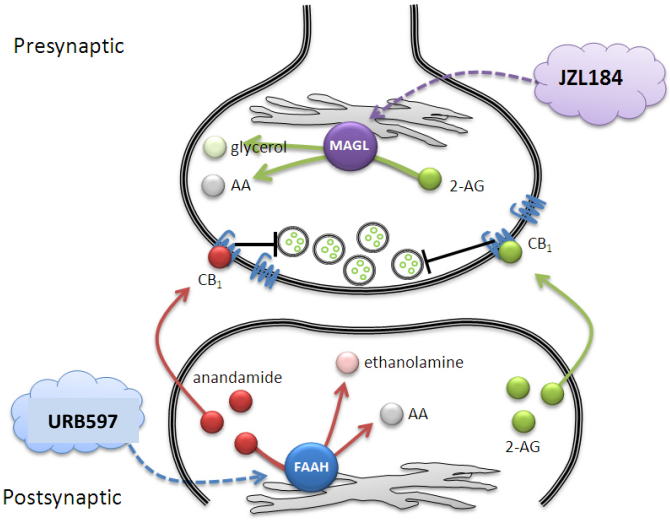


Figure 12. **Endocannabinoid enhancement by inhibiting the enzymes responsible for its metabolism.** Schematic diagram showing the inhibition of FAAH by URB597 occurs at a postsynaptic level, whereas the inhibition of MAGL by JZL184 is located in the presynaptic neuron. The increase of anandamide or 2-AG produces anxiolytic-like effects *(Ahn et al., 2009)*.

The cannabinoid receptors involved in the regulation of anxiety by the endocannabinoid system has also been identified using pharmacological and genetic tools. The use of CB1R antagonists and CB1R knockout (CB1RKO) mice *(Table 4)* mainly show an increase in the anxiogenic-like responses in different behavioral paradigms (Haller et al., 2002, Martin et al., 2002). The use of conditional knockout mice

has recently allowed demonstrating that the anxiolytic-like effects of cannabinoids are mediated through CB1R specifically located in glutamatergic neurons, whereas CB1R located in the GABA-ergic neurons are responsible for the anxiogenic-like responses (Rey et al., 2012). Moreover, other neurotransmitter systems, such as the opioid system, have been involved in the modulation of the anxiety-like responses by cannabinoids (Valjent et al., 2002, Berrendero and Maldonado, 2002).

Drug or Mutant	Species	Drug	Doses (mg/kg)	Test	Effects	References
CB1R agonists	Rats Mice	THC CP55,940	0.3-10	EPM OF LDB	High doses are anxiogenic whereas low doses are anxiolytic	(Rey et al., 2012, Puighermanal et al., 2013)
CB2R agonists	Mice	JWH133	1-10	EPM	Anxiolytic	(Busquets-Garcia et al., 2011)
CB1R antagonists	Rats Mice	SR141716 (Rimonabant) AM251 AM281	0.3 – 10	EPM ZM LDB OF	High doses are anxiogenic whereas low doses have no effect or anxiolytic	(Haller et al., 2002, Martin et al., 2002)
CB1RKO	Mice			EPM LDB	Normal or mild anxiety	(Martin et al., 2002)
Anandamide reuptake inhibitor	Rats Mice	AM404	0.5-10	EPM LDB	Anxiolytic	(Patel and Hillard, 2006)
FAAH inhibitors	Rats Mice	URB597	0.05-1	EPM ZM	Anxiolytic	(Kathuria et al., 2003, Moreira et al., 2008)
MAGL inhibitors	Rats Mice	JZL184	8-16	EPM BM	Anxiolytic	(Sciolino et al., 2011, Busquets-Garcia et al., 2011)
FAAHKO	Mice			EPM	Anxiolytic	(Cippitelli et al., 2008)

Table 4. **Summary of different studies that have examined the anxiety-like responses produced by different endocannabinoid system modulators or genetic models.** EPM, elevated-plus maze; OF, open field; LDB, light/dark box; ZM, zero maze; MBT, marble burying test.

Recently, the role of CB2R in endogenous anxiety-like responses has been proposed (Garcia-Gutierrez and Manzanares, 2011) suggesting a novel target of the endocannabinoid system to modulate anxiety-related behaviors.

1.3.3. Nociception

The analgesic properties of exogenous and endogenous cannabinoids are widely documented and the endocannabinoid system has been localized in multiple neural regions involved in nociceptive responses (Hohmann, 2002). Cannabinoids exert antinociceptive effects by acting at three different levels *(Figure 13)*. It is known that the administration of cannabinoid agonists at supraspinal level into various brain regions (periaqueductal gray, thalamus or amygdala) produces antinociceptive effects (Walker and Hohmann, 2005). On the other hand, behavioral, electrophysiological and neurochemical studies have demonstrated that cannabinoids also act at the spinal level to modulate pain (Guindon and Hohmann, 2009). Finally, peripheral and local antinociceptive actions of cannabinoids have been demonstrated in several animal pain models (Walker and Hohmann, 2005).

Different studies provided support for a physiological role of endocannabinoids in the control of pain, as revealed by the hyperalgesia obtained after blocking CB1R (Guindon and Hohmann, 2009). Moreover, there is evidence that noxious stimuli produce an enhancement of the endocannabinoids levels in different structures related to nociceptive control (Jhaveri et al., 2007). Although it is well established that these endocannabinoids produce antinociception by

activating the CB1R, it is also known that CB2R play a crucial role in the regulation of pain. Indeed, CB2R have been involved in the central immune responses leading to neuropathic and osteoarthritic pain (Racz et al., 2008a, Racz et al., 2008b, La Porta et al., 2013).

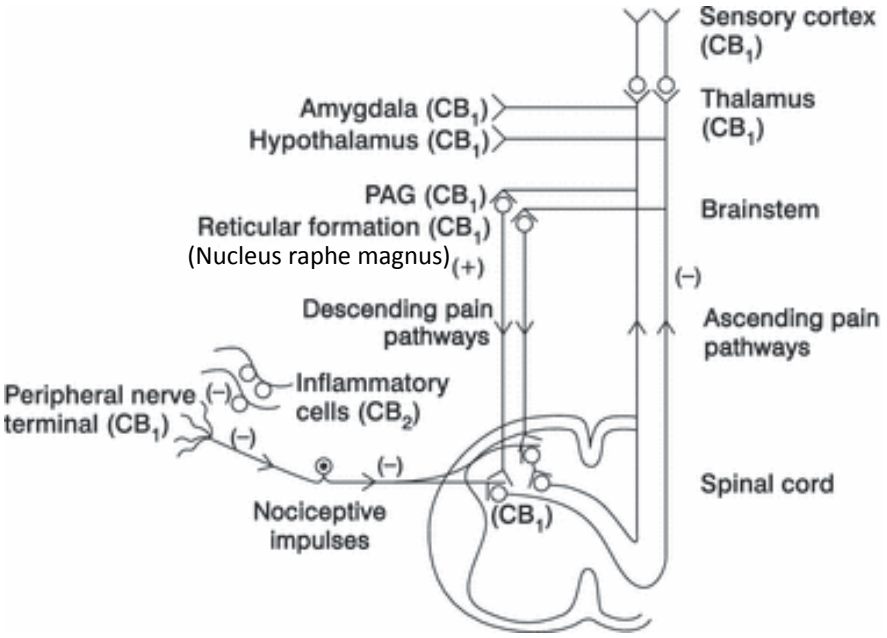


Figure 13. **Cannabinoid receptors in pain processing pathways.** CB1R are present on peripheral nerve terminals, dorsal horn spinal cord and pain processing pathways in the brain. Their activation inhibits the excitatory ascending pain pathways, modifies pain integration and stimulates activity in descending pain pathways. CB2R are present in blood cells associated with inflammation and may modulate pain initiation at the site of tissue injury *(Ashton and Moore, 2011).*

The exogenous administration of endocannabinoids or specific inhibitors of the enzymes responsible for their degradation or re-uptake produces antinociception in different paradigms of acute, inflammatory or nerve injury-induced pain (Guindon and Hohmann, 2009, Hohmann and Suplita, 2006, Buxbaum, 1972, Hutcheson et al.,

1998, Martin, 1985). However, the magnitude of this antinociceptive effect may differ depending on the test, the endocannabinoid administered, the dose and the context. On the other hand, recent studies using genetic disruption of FAAH or MAGL show a CB1R-dependent antinociception in different assays (Ahn et al., 2009, Lichtman et al., 2004). However, more research is needed to further characterize the role of this system in each type of pain.

1.3.3. Neuronal excitability

The endocannabinoid system provides protection against hyperexcitability and acute seizures, and it is neuroprotective against excitotoxicity via CB1R-dependent mechanisms (Marsicano et al., 2003). In animal models of epilepsy, CB1R-agonists have strong anti-epileptic effects (Deshpande et al., 2007, Naderi et al., 2008). In humans, these effects are less clear and not well studied. Indeed, some studies suggest that cannabis may have anti-epileptic effects (Consroe, 1998), whereas others found it ineffective or proconvulsant when is consumed for recreational use (Lutz, 2004).

On the other hand, the endocannabinoid system has also been implicated in the process of epileptogenesis (Hofmann and Frazier, 2011). Interestingly, early administration of CB1R antagonists was anti-epileptogenic and prevented the development of epilepsy (Chen et al., 2007). In the hippocampus, a key region involved in epilepsy (Tellez-Zenteno and Wiebe, 2011), CB1R are present on both excitatory glutamatergic and inhibitory GABA-ergic neurons (Hoffman et al., 2010, Hoffman and Lupica, 2000). Two different hypotheses have been postulated to explain the role of the

endocannabinoid system in epilepsy. Firstly, an acute on-demand activation of CB1R on glutamatergic neurons could occur as an anti-epileptic mechanism. Secondly, a long-term upregulation of CB1R on GABA-ergic neurons could lead to hyperexcitability and epileptic seizures (Goffin et al., 2011, Hofmann and Frazier, 2011). The better understanding of the role of endocannabinoid system in seizure formation could serve to improve therapeutic approaches in neuronal disorders accompanied by epileptic episodes.

1.4. Potential therapeutic applications of the endocannabinoid system

Despite the public concern related to the important negative consequences of the recreational use of marijuana and its derivatives, cannabinoids are gaining more weight in modern medicine due to their promising therapeutic properties. Thus, the activation of the endocannabinoid system results in anti-inflammatory effects, produces antinociception and sedation, increases appetite, improves mood, decreases intraocular pressure, reduces emesis, exerts bronchodilatation, neuroprotection and antineoplastic effects (Pertwee, 2012). Moreover, recent works demonstrate that deregulation of the endocannabinoid system is involved in several neuronal disorders such as depression and anxiety (Hillard et al., 2012), schizophrenia (Saito et al., 2012), Alzheimer's disease (Martin-Moreno et al., 2012), Huntington's disease (Sagredo et al., 2012) and autism spectrum disorders such as fragile X syndrome (Jung et al., 2012). In this section we will describe the current state of the endocannabinoid system modulation as a therapeutic strategy as well as the limitations of its potential clinical use.

1.4.1. Cannabinoid antagonists

The design of cannabinoid receptor antagonists as pharmacotherapeutics that decrease the endocannabinoid transmission has gained interest in the research community. Interestingly, a hyperactive endocannabinoid system appears to contribute to the etiology of several disease states, including obesity or cardiometabolic diseases.

1.4.1.1. Therapeutic relevance

Cannabinoid receptor antagonists have been proposed as a therapeutic tool for disorders that involve a pathologically endocannabinoid system activity, including overweight/obesity, cardiometabolic problems and drug addiction (Vemuri et al., 2008, Di Marzo and Petrosino, 2007, Janero and Makriyannis, 2007). The possible use of these antagonists on other neural diseases, such as Alzheimer's disease, head trauma and senile dementia has also been postulated (Janero and Makriyannis, 2009, Kunos et al., 2009). Moreover, the recent involvement of the endocannabinoid system in other neuronal disorders, such as fragile X syndrome or schizophrenia, suggests that more research will be needed to clarify the potential therapeutic profit of cannabinoid antagonists in these diseases (Jung et al., 2012, Ferretjans et al., 2012).

The increasing global obesity epidemic makes the search of novel approaches for the treatment of overweight/obesity a priority for the pharmaceutical industry. Different mouse models and human data demonstrate the involvement of an enhanced endocannabinoid signaling in the etiopathogeny of obesity (de Kloet and Woods, 2009).

Rimonabant, a CB1R antagonist/inverse agonist, was the first drug used to treat obesity and cardiometabolic diseases with clinical effectiveness (Fernandez and Allison, 2004). Unfortunately, rimonabant was not approved by the Food and Drug Administration (FDA) and suspended by the European Medicines Evaluation Agency (EMEA) due to severe side effects (Taylor, 2009). However, its initial success in clinical trials led many pharmaceutical companies to develop comparable drugs (Janero and Makriyannis, 2009). Thus, taranabant, otenabant and surinabant (Janero and Makriyannis, 2009) also reached an advanced clinical development stage, although they have also been disrupted because of their side effects. Nevertheless, alternative approaches are being actively pursued to attenuate pathologically hyperactive endocannabinoid system activity. Two novel strategies have raised particular interest: peripheral CB1R antagonists and pure neutral CB1R antagonists *(Figure 14)*. Peripheral CB1R antagonists with a limited penetration across the blood-brain barrier reduce obesity-associated cardiometabolic risk with improved safety over rimonabant (Kunos et al., 2009). On the other hand, neutral antagonists that bind CB1R without eliciting changes in constitutive or intrinsic cellular endocannabinoid signaling (Greasley and Clapham, 2006, Bond and Ijzerman, 2006) may be a promising therapeutic tool against pathologies with an overactivated signaling through CB1R (Pagotto et al., 2006, Duffy and Rader, 2007, Kunos et al., 2008, Perkins and Davis, 2008). These neutral antagonists could have a better safety profile than the previous antagonists with intrinsic inverse agonist properties.

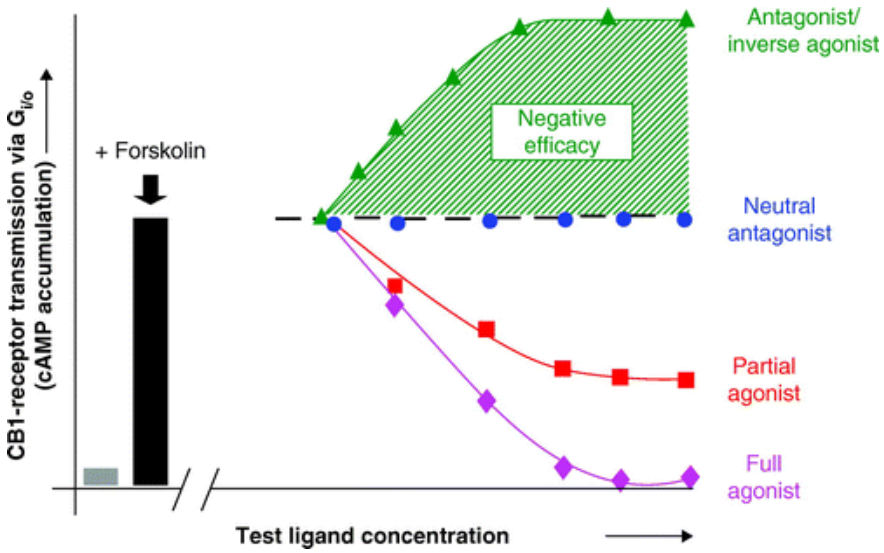


Figure 14. **Schematic representation of the pharmacological modes of action of various ligands on constitutive CB1 receptor activity.** In the absence of agonist, the low-level constitutive signal transmission (grey bar) is markedly potentiated by forskolin (arrow, black bar), which experimentally activates adenylyl cyclase and increase cAMP. A CB1R agonist activates the inhibitory G protein reducing basal cAMP formation. An antagonist/inverse agonist elicits a 'negative efficacy' response to potentiate constitutive signaling and cause cAMP accumulation. In marked distinction, identical concentrations of a neutral antagonist do not affect constitutive CB1R activity *(Janero and Makriyannis, 2009).*

Although less research has been done, preliminary data suggest that CB2R antagonist/inverse agonists may also have therapeutic relevance. CB2R have a crucial role in mediating immune and inflammatory responses (Mackie, 2008, Pertwee, 2006). In addition, several groups have demonstrated in animal models that CB2R antagonists have beneficial effects in inflammatory disorders, arthritic bone damage and experimental autoimmune encephalomyelitis (Cheng and Hitchcock, 2007, Ashton and Glass, 2007). Interestingly, it has been recently demonstrated that the lack of CB2R-mediated responses

protects mice from both age-related and diet-induced insulin resistance, suggesting that these receptors may have also therapeutic properties in obesity and insulin resistance (Agudo et al., 2010). However, the therapeutic use of these compounds must await initial clinical testing and the translation to humans.

1.4.1.2. Limitations

Rimonabant was not approved by the FDA in the USA and it was suspended in Europe although causing clinically significant weight loss and reduction of different cardiometabolic risk factors. The safety of CB1R antagonists is therefore in doubt because of serious side effects that include depression, suicidal thoughts and anxiety (McLaughlin, 2012) *(Table 5)*. Therefore, future CB1R antagonists must include screening for depression-like and anxiety-like effects. In addition, a possible enhancement in the incidence of seizures in vulnerable patients must also be considered in future compounds.

Promising expectatives had appeared at the beginning because the adverse effects (nausea, dizziness and diarrhea) associated with rimonabant treatment were not serious in the first clinical trials (RIO and STRATUS). However, an increased incidence of depression-related mood disorders and anxiety was also noted in the rimonabant group (Van Gaal et al., 2005). In 2007, upon a review of several clinical studies, the FDA advisory committee voted unanimously to not to recommend rimonabant for use in obesity due to severe psychiatric adverse effects (depressed mood disorders, anxiety and suicide ideation) (Christensen et al., 2007). Moreover, another clinical trial, STRADIVARIUS, that did not excluded subjects with prior

psyquiatric disorders, concluded that rimonabant was worse in a subject population with an initially higher incidence of psychiatric symptoms (Rumsfeld and Nallamothu, 2008). In addition, other CB1R antagonists such as taranabant, otenabant or surinabant were also suspended due to similar adverse effects (Janero and Makriyannis, 2009).

	RIO-EUROPE	RIO-Lipids	RIO-North America	RIO-Diabetes	STRADIVARIUS	SERENADE
Depression	-	2,3%	2,1%	2,1%	6,4%	5,1%
Anxiety	-	4,9%	4,0%	2.0%	6,2%	2,2%
Insomnia	-	3,8%	1,4%	-	3,2%	-
Aggression	-	0,6%	-	-	-	-
"Serious Psychiatric disorders	1,2%	-	3,9%	-	1,9% (15% non-serious)	-

Table 5. **Side effects of different clinical trials using Rimonabant** *(Taylor, 2009).*

1.4.2. Cannabinoid agonists

The activation of cannabinoid receptors has been used for clinical and recreational purposes over thousands of years. Although several positive effects results from the endocannabinoid system activation *(Table 6)*, clear negative effects are also induced by cannabinoid agonists.

Indication	Compound + Disease
Spasticity	Dronabinol/cannabis cigarettes/cannabis extract in multiple sclerosis Dronabinol/nabilone in paraplegia
Nausea and vomiting due to cytostatics	Dronabinol/cannabis cigarettes/cannabis extract/nabilone in chemotherapy related nausea and vomiting
Loss of appetite/ weight loss	Dronabinol/cannabis cigarettes in HIV/Aids Dronabinol/cannabis extract in various tumor diseases Dronabinol in Alzheimer's disease
Chronic pain	Dronabinol/nabilone/cannabis extract/cannabis cigarettes in neuropathic pain or pain in multiple sclerosis Dronabinol/nabilone/cannabis cigarettes/cannabis extract in chronic pain (cancer, rheumatism, fibromyalgia)

Table 6. **Overview of controlled trials of cannabis medications for established indications** *(Grotenhermen and Muller-Vahl, 2012).*

1.4.2.1. Therapeutic relevance

There is convincing evidence that the direct activation of CB1R has potential therapeutic applications in several disorders, including cancer, neuropathic and inflammatory pain, multiple sclerosis, intestinal disorders, post-traumatic stress disorder, traumatic brain injury, hemorrhagic, septic and cardiogenic shock, hypertension, atherosclerosis, Alzheimer's disease and Parkinson's disease (Pertwee, 2005). In this regard, compounds that inhibit the cellular uptake or metabolism of endocannabinoids are of particular interest for therapeutic purposes (Pertwee, 2005, Petrosino and Di Marzo, 2010). Overall, the goal in the field is to maximize the beneficial therapeutic effects and/or minimize the unwanted side effects of the activation of the endocannabinoid system.

Different licensed medicines that activate directly the cannabinoid receptors have already been developed *(Table 6)* (Pertwee, 2009, Pertwee, 2012). THC, also named as Dronabinol or Marinol, and its synthetic analogue, Nabilone, have been used for suppressing nausea and vomiting in chemotherapy, or as appetite stimulants in patients with AIDS (Pertwee, 2012). Another example is Sativex that is a mixture of THC and the non-psychoactive plant cannabinoid, cannabidiol. Sativex was accepted in Canada as a symptomatic relief of neuropathic pain in multiple sclerosis and as adjunctive analgesic treatment for adult patients with advanced cancer. Moreover, it was also approved by European Medicines Agency (EMA) and licensed in UK and Canada for the treatment of spasticity in multiple sclerosis and has recently been approved in other countries, including Spain (Pertwee, 2012).

In order to minimize the central adverse effects, there are peripherally restricted compounds, studied mainly for pain relief, that activate the cannabinoid receptors outside the CNS. Some examples are the naphthalene-1-yl-(4-pentyloxynaphtalen-1-yl)-methanone (potent CB1R and CB2R agonist) (Dziadulewicz et al., 2007) and the "compound A" (CB1R and CB2R agonist), among other drugs. These compounds have analgesic properties in different animal models of acute, inflammatory and neuropathic pain (Yu et al., 2010, Pertwee, 2012). Another strategy to minimize the central side effects is to inject the cannabinoid agonists locally in specific regions containing cannabinoid receptors. For example, the direct activation of both CB1R and CB2R in the skin or in the spinal cord produces antinociception (Hama and Sagen, 2011, Potenzieri et al., 2008). This

local administration could also be indicated to reduce tumors (Gu et al., 2011, Khasabova et al., 2011). Moreover, CB2R agonists have gained attention because most of the adverse effects of cannabinoid agonists are due to central CB1R activation. These agonists have several potential therapeutic effects in different disorders, including analgesia, Parkinson's and Huntington's diseases, anxiety, neurodegeneration, neuroinflammation, impulsivity, cocaine dependence, cancer and stroke, among others (Pertwee, 2012). Finally, as in the case of Sativex, the coadministration of a second drug with the cannabinoid agonist could also improve its effects (Pertwee, 2012). Nowadays, there is an urgent need to implement some of these potential new strategies into clinical trials.

The increase of endocannabinoids by specific inhibitors of their metabolism has been used as an alternative of direct cannabinoid agonists for therapeutic purposes *(Table 7)*. Thus, the inhibition of FAAH reduces the emesis through CB1R and TRPV1, induces food intake, shows efficacy against inflammatory and neuropathic pain, has been proposed to be of interest in gastrointestinal and hepatic disorders, presents anti-dyskinetic effects in experimental models of Parkinson's disease and produces anxiolytic-like responses, among others (Petrosino and Di Marzo, 2010). On the other hand, different experimental models have shown that the specific inhibition of the MAGL counteracts inflammatory and neuropathic pain, reduces inflammation, produces anxiolytic-like effects, has efficacy in head trauma and shows effectiveness in other neuronal disorders such as Alzheimer's disease or multiple sclerosis, among others (Ligresti et al., 2009). Unlike exogenously administered cannabinoid agonists, the

endocannabinoids that are produced endogenously may act in a site- and time-specific manner to minimize the side effects. However, clinical data with any of these compounds have not been yet provided. Therefore, the final development of clinical therapies from such inhibitors still requires substantial effort to maximize their efficacy and reduce several potential off-targets.

Inhibitor	Target	Possible therapeutic use	Possible indications
AA-5-HT	FAAH	Cancer, inflammatory diseases, analgesia, anxiety, post-natal hippocampal damage	Blockade of proliferation/tumor growth, colitis induced by sulfonic acids, stress-induced analgesia, acute inflammation, chronic pain, anxiety-like responses
OL-135	FAAH	Analgesia, post-traumatic stress syndrome, obsessive-compulsive disorders and dermatological disorders	Acute and chronic pain, pruritus
Org-231295	FAAH	Analgesia, inflammatory diseases, depression, anxiety, emesis, dermatological disorders, neurodegenerative disorders	Inflammatory and neuropathic pain, osteoarthritis, bowel diseases, colitis, depression-like phenotypes, anxiety-like responses, lithium/cisplatin/nicotine induced nausea, pruritus and Parkinson's disease
PF-3845	FAAH	Analgesia	Inflammatory pain
CAY-10401	FAAH	Invasion of prostate carcinoma cells	Migration of cells
JNJ-1661010	FAAH	Analgesia	Inflammatory and neuropathic pain
URB-602	MAGL	Analgesia	Inflammatory pain
OMDM-169	MAGL	Analgesia	Acute pain
JZL-184	MAGL	Analgesia	Acute pain

Table 7. **Possible therapeutic use of FAAH and MAGL inhibitors as suggested by preclinical studies** *(Petrosino and Di Marzo, 2010).*

1.4.2.2. Limitations

There are no doubts regarding the therapeutic potential of cannabinoid agonists. However, both cannabis derivatives and synthetic cannabinoids present several side effects that limit the medicinal potential of these compounds (Hagenbach et al., 2007). Drug users perceive pleasurable and relaxing effects after acute cannabis consumption. However, the subjective feeling produced by cannabinoids can lead to dysphoria, anxiety-like effects or panic when these effects are not expected. Other acute effects of cannabinoids that can difficult their therapeutic applications are memory impairment, reductions in psychomotor and cognitive performance, disordered perception of the passage of time and euphoria (Grotenhermen and Muller-Vahl, 2012).

The side effects of chronic exposure to cannabinoids become very important taking into account that these therapeutic substances should be administered chronically to exert most of their effects. High consumption of cannabis has long-term consequences on cognitive performance (Solowij and Battisti, 2008). Nevertheless, it has been demonstrated that only extremely high consumption at levels hardly ever used for therapeutic purposes leads to irreversible cognitive impairments (Pooyania et al., 2010). However, children and adolescents are particularly sensitive to these cognitive effects and advisability of a treatment with cannabinoids in this age must be weighed up very carefully (Grotenhermen and Muller-Vahl, 2012).

Cannabis consumption may also induce psychotic symptoms in vulnerable individuals. In addition, a metanalysis revealed that there

was an increased risk of any psychotic outcome in individuals who had ever used cannabis (odds ratio=1.41). Moreover, a greater risk was found in people who used cannabis most frequently (odds ratio=2.09) (Moore et al., 2007). In agreement, recent studies demonstrated that cannabis exposure double the risk to suffer these psychotic symptoms (Saito et al., 2012, Bugra et al., 2012). It also may produce panic and anxiety-like responses (Di Forti et al., 2007, Laqueille et al., 2008). Other physical effects induced by cannabinoids are tiredness, dizziness, orthostatic hypotension, obnulations, dry mouth, reduced lacrimation, muscle relaxation and increased appetite (Grotenhermen and Muller-Vahl, 2012). Importantly, one of the side effects of cannabinoids that most frequently appears is the cardiovascular response that can lead to tachycardia and in some few cases to myocardial infarction. (Tormey et al., 2012). Finally, withdrawal symptoms occur in heavy users of cannabis after abrupt cessation of consumption, such as uneasiness, irritability, sleeplessness, increased perspiration and loss of appetite (Cooper and Haney, 2009). Nevertheless, these withdrawal symptoms rarely represent a problem in the controlled medical administration of cannabinoids (Ekert et al., 1979, Grotenhermen and Muller-Vahl, 2012). It is important to underline that another undesirable effect of chronic cannabinoid treatment is the development of tolerance that will be described in the next section. Therefore, a priority in cannabinoid research has been to further investigate these side effects in order to understand the mechanisms that underlie each negative effect and to develop strategies to minimize them.

1.4.2.3. Tolerance and physical dependence

Tolerance to most of the central and peripheral effects of cannabinoids develops when the administration becomes chronic (Dewey, 1986, Kaminski et al., 1992, Maldonado and Rodriguez de Fonseca, 2002). In humans, there is evidence of pharmacological tolerance for most of the effects of cannabis (Jones et al., 1981, Hollister, 1986). In contrast, chronic cannabinoid exposure also produces the development of sensitization (Rubino et al., 2003) that increases the drive and motivation for the substance.

In laboratory animals, the degree and time-course of tolerance are dependent on the animal species, the type of ligand, the dosage, the duration of treatment, the measure employed to determine tolerance, and the pharmacokinetic and pharmacodynamic properties of ligands. Thus, tolerance after repeated cannabis administration in analgesia, motor inhibition or hypothermia occurs typically after 3-7 days (Gonzalez et al., 2005). However, there is controversy regarding the tolerance to other cannabinoid effects, such as the memory impairment or anxiety-like responses. Some studies demonstrated a lack of tolerance for the amnesic-like effects induced by cannabinoids (Ferraro and Grilly, 1973, Heishman et al., 1997). In contrast, others showed the development of tolerance to these cognitive effects after several weeks of cannabis administration (Hampson et al., 2003). In addition, tolerance could be also dependent on the different sensitivity of brain structures that regulate different functions. Thus, some studies have demonstrated an earlier tolerance to the analgesic effects than to the hypothermic or cataleptic responses (Spina et al., 1998, Sim-Selley, 2003). There are different hypotheses to explain these

region-dependent effects: a region-dependent pattern of activation induced by cannabinoids (Whitlow et al., 2002, Whitlow et al., 2003), differences in the coupling of CB1R to GTP-binding proteins (Breivogel et al., 1997, Steindel et al., 2013) or distinct proportion of CB1R in the different neuronal types (Kawamura et al., 2006).

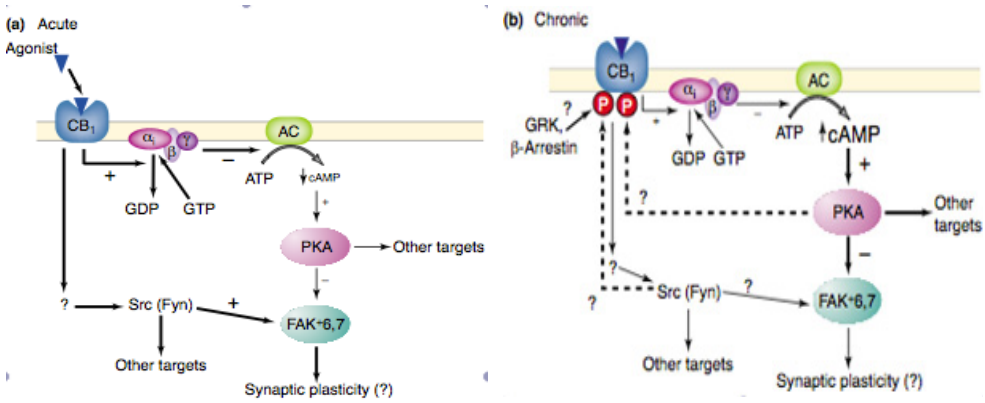


Figure 15. **CB1R signaling pathways implicated in cannabinoid tolerance.** A) Acute activation of CB1R triggers the Gi/o proteins inhibiting the adenylyl cyclase activity. The decreased cAMP levels result in attenuation of PKA activity and modulation of neuronal focal adhesion kinase. CB1R activation also modulates other targets. B) Chronic exposure to the agonist leads to uncoupling of the CB1R from Gi/o, possibly via G-protein-coupled receptor kinase-arrestin-mediated desensitization and down-regulation. The levels on cAMP are also enhanced by the hyperactivation of adenylyl cyclase, which results in increased PKA activity. PKA could modulate different targets that maintain the cannabinoid tolerance *(Martin et al., 2004).*

Additionally, an important characteristic of cannabinoid signaling adaptation is the variation in the magnitude and rate of CB1R desensitization and down-regulation in different brain areas (McKinney et al., 2008, Breivogel et al., 1997). Different mechanisms

have been proposed to understand the CB1R desensitization after THC or synthetic cannabinoids exposure *(Figure 15)*: activation of several proteins involved in the internalization of the CB1R such as beta-arrestin (Rubino et al., 2006), G-protein-coupled receptor-associated sorting protein 1 (Martini et al., 2010) or specific kinases that phosphorylate serine residues in the structure of the receptor (Lee et al., 2003). However, further studies will clarify the mechanisms that underlie tolerance and CB1R down-regulation because this is an important caveat for the therapeutic use of cannabinoids.

Finally, a withdrawal response that occurs upon cessation of cannabinoid agonist administration has been reported in humans and animals (Lichtman and Martin, 2005, Cooper and Haney, 2009). In laboratory animals, the main behavioral responses observed after a spontaneous or CB1R antagonist-precipitated withdrawal are writhings, wet dog shakes, sniffings, front paw tremors, genital lickings, erection, ataxia, ptosis, diarrhea, mastication, decreased grooming and piloerection (Tanda and Goldberg, 2003) *(Table 8)*. In humans, the symptoms of cannabinoid withdrawal are anger, irritability, anxiety, decreased appetite, weight loss, restlessness, disturbances in sleep and cannabis craving. However, some of these effects depend on the doses consumed, the levels of THC in the preparations and the frequency of consumption of this drug (Cooper and Haney, 2009).

Species	Agonist	Quantified withdrawal signs
Dog	THC	Withdrawal from human contact, trembling, shaking, shivering, exaggerate response to auditory or visual stimuli, vomiting, diarrhea
Rat	THC	Facial rubbing, wet dog shakes, forepaw fluttering, grooming, horizontal activity, vertical activity, mouth movement, scratching, licking, suppression of food-reinforced operant responding.
	CP55,940	Turning, mastication, digging
	WIN 55,212-2	Wet dog shakes, facial rubs
	HU-210	Global withdrawal score
	Anandamide	Ptosis, hunched position, wet dog shakes, piloerection, forepaw fluttering
Mouse	THC	Wet dog shakes, facial rubbing, ataxia, hunched position, tremor, ptosis, piloerection, mastication, sniffing

Table 8. **Rimonabant-precipitated withdrawal effects in cannabinoid-dependent animals** *(Lichtman et al., 2002).*

2. Cannabis and memory

It has been widely demonstrated that cannabis intake causes memory impairment in human and laboratory animals. The role of the endocannabinoid system in memory has been extensively studied in this thesis.

2.1. Neuroanatomical and neurobiological substrates of memory

Memory is a brain function that classifies, encodes, stores, and recovers different information relevant for the subject (Squire, 1986, Kandel, 2001). Memory can be divided in two major groups *(Figure 16):* declarative and non-declarative. Declarative or explicit memory is defined as the conscious memory for facts and events and is acquired with few exposures to the material to be learned. It can be classified into episodic memory (personal events) and semantic memory (general facts) (Squire, 1992, Squire and Zola, 1996). This type of memory is mainly controlled by the medial temporal lobe, including the hippocampus, the entorthinal, perirhinal and parahipocampal cortices (Moscovitch et al., 2006). On the other hand, non-declarative or implicit memory, which is more complex and involved different brain areas, consists in procedural memory for habits or skills and usually requires an extensive acquisition phase (Schacter and Cooper, 1993).

The time scale could also be used to classify memory *(Figure 16)*. During every moment of an organism's life, sensory information is being taken in by sensory receptors and processed by the nervous system. This first impression is the sensory memory that allows

individuals to retain impressions of sensory information after the original stimulus has ceased. In a second step, working memory permits to remember the current state of a plan that that somebody is executing. For example, while doing mental arithmetic is necessary to remember intermediate results. This information lasts from milliseconds to minutes and it is quickly lost if not constantly refreshed and is controlled mainly by the prefrontal cortex (Cowan, 2001, Tetzlaff et al., 2012). Secondly, the short-term memory lasts from minutes to days in humans and from minutes to 3-4 hours in rodents. Although depends on the context and the memory test, the brain region mostly associated is the hippocampus. This type of memory is still susceptible to perturbations (Walker et al., 2003, Kumaran, 2008). Finally, long-term memory is the memory type with the longest time scale, from days to years in humans and from hours to days in rodents. This memory is created with the involvement of different brain regions and synaptic and plastic changes are required (Xu et al., 2009, Frankland and Bontempi, 2005, Ziv and Ahissar, 2009).

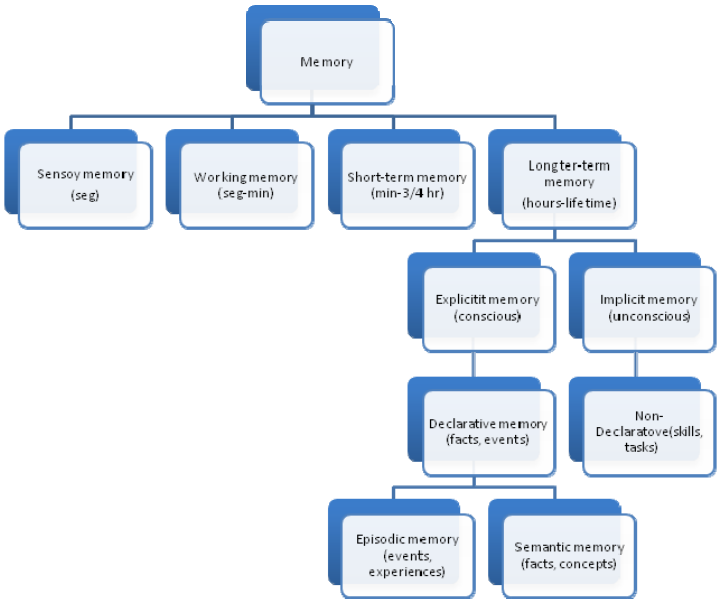


Figure 16. **Schematic representation of the different memory types and the time scale of these different types.**

The hippocampus is the major component of the brain of vertebrates involved in memory formation. It belongs to the limbic system and plays a key role in the consolidation of information, especially the declarative memory. The dentate gyrus, CA1 and CA3 areas mainly compose this brain region *(Figure 17)*. The flow of information into the hippocampus starts in the entorhinal cortex that is the main input of the hippocampus that also receives information from the parahippocampal and perirhinal cortices. Thus, the entorhinal cortex projects to the dentate gyrus and CA3 via the perforant pathway (layer I), whereas it projects directly to CA1 and subiculum through layer III. CA3 also projects to CA1 through the Schaffer-collaterals (Witter and Amaral, 1991). The hippocampal CA1 pyramidal neurons receive excitatory inputs by the perforant path (enthorhinal cortex) and by the Schaffer-collaterals (CA3) (Amaral and Witter, 1989, Steward and

Scoville, 1976). Both inputs to the pyramidal neurons are crucial for memory formation in the hippocampus (Remondes and Schuman, 2002, Remondes and Schuman, 2004).

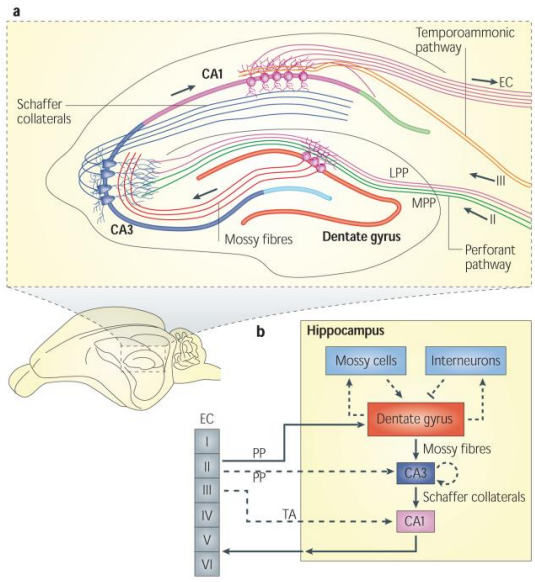


Figure 17. **An illustration of the hippocampal circuitry (A) and a diagram of the hippocampal neural network (B).** Solid arrows depict the traditional excitatory trisynaptic pathway. The axons of layer II neurons in the entorhinal cortex project to the dentate gyrus through the perforant pathway, including the lateral perforant pathway and medial perforant pathway. The dentate gyrus sends projections to the pyramidal cells in CA3 through mossy fibers. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals. CA1 pyramidal neurons send back-projections into deep-layer neurons of the enthorhinal cortex. CA3 also receives direct projections from enthorhinal cortex layer II neurons through the perforant pathway. CA1 receives direct input from entorhinal cortex layer III neurons through the temporoammonic pathway. The dentate granule cells also project to the mossy cells in the hilus and hilar interneurons, which send excitatory and inhibitory projections back to the granule cells, respectively *(Remondes and Schuman, 2004).*

The brain encodes and stores information controlling the complex signaling network that modulates translation and protein synthesis. Changes at the synaptic level are crucial for memory processes. A putative mechanism, the "synaptic tagging" *(Figure 18)* has been proposed to understand how memories are stored consisting in a local and persistent protein modification that serves as a marker for the synapse that will be modified (Redondo and Morris, 2011, Lesburgueres et al., 2011). Local protein synthesis in neuronal dendrites is notably important for the synaptic plasticity that occurs during memory storage (Barco et al., 2006).

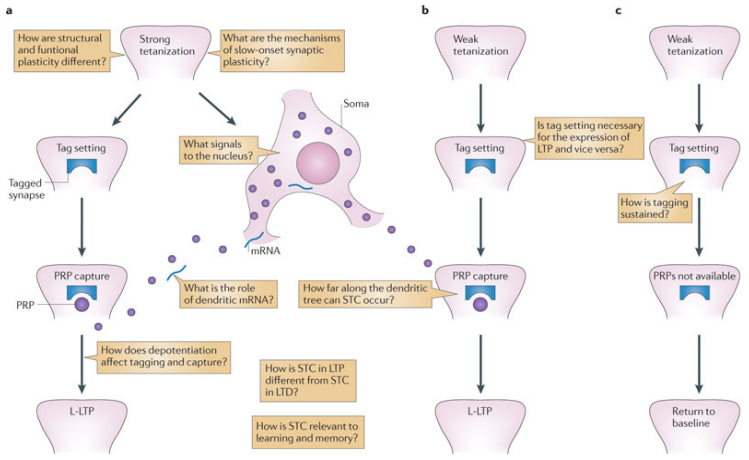


Figure 18. **Schematic representation of the synapting tagging theory.** A) As originally proposed, the strong tetanization of a synaptic pathway leads to two dissociable events: local tag setting and the synthesis of diffusible plasticity-related proteins. The plasticity-related proteins are then captured by tagged synapses, and this is necessary for the maintenance of late-long-term potentiation. B) A weakly stimulated set of synapses that has access to the plasticity-related proteins will also succeed in maintaining late-long-term potentiation. C) Without the availability of plasticity-related proteins, the receptive state (tagging) of the synapses will fade and late-long-term potentiation will not be sustained *(Barco et al., 2006).*

The mechanisms that underlie this protein synthesis are very complicated. However, the mTOR signaling pathway and its downstream targets are key regulators of protein translation and synthesis. Thus, both pharmacological and genetic approaches indicate that mTOR plays a crucial role in memory processes because its inhibition produces memory impairment (Antion et al., 2008b, Parsons et al., 2006, Banko et al., 2007). In addition, the training of several memory tasks modulates this pathway (Parsons et al., 2006, Kelleher et al., 2004). Interestingly, some neuronal disorders accompanied by cognitive decline have been recently associated to an overactivation of the mTOR pathway (Sharma et al., 2010a, Ma et al., 2010).

2.2. Behavioral mouse models to study memory

Memory is a function extensively studied in animal models and different behavioral paradigms are available to evaluate memory performance *(Box 1)*. Memory is evaluated with different mazes and training schedules from only one session to more complex operant learning tasks. Positive reinforces, such as food, sweetened water or novelty, as well as negative reinforces, such as water immersion, intense light, electric shock, or a loud noise, have been used in these memory paradigms (Sharma et al., 2010b). Multiple tests have been developed to mimic the natural behavior of mice, including T- and Y-maze alternation task (Gerlai, 1998b), novel object recognition test (Dere et al., 2007), and social recognition task (Thor et al., 1982), among others. On the other hand, other models require an aversive component, such as passive avoidance task, fear conditioning and

conditioned taste aversion (Gerlai, 1998a, Lee and Silva, 2009), among others.

Morris water maze

Spatial learning and memory task known to depend on the hippocampus. Animals swim in a pool of water to find the location of a submerged platform just beneath the surface of the water. There are different cues and strategies to scape the water, including spatial cues around the pool. Animals are trained during several days and the time/path length they take to find the platform is the learning index. There are different alternatives of this task. For example, a test in which the platform is removed and the mice are allowed to search for it. In this case, the time that the animal is looking for the platform in the quadrant where it was placed before is the learning index.

Novel object recognition task

Non-aversive and non-spatial task that requires hippocampal function. It is based in the innate tendency to explore the novelty. Animals are allowed to freely explore two objects in a maze during a training session. In the test session, a novel object replaces one of the objects. A discrimination index is calculated and the longer mice explore the novel object, the higher is the discrimination index indicating good memory.

Radial arm maze

Spatial learning task with various versions. The apparatus has several arms (most commonly eight) that can be baited with food pellets at the end. Food-deprived animals are allowed to enter the arms and search for hidden food. Different variants of this task are done blocking or giving access to the different arms with or without food. Depending on the version, the hippocampus or the prefrontal cortex are the most important brain regions in this model.

Fear conditioning

Aversive learning task in which animals associate a non-aversive conditioned stimuli, such as a tone or context, with an aversive unconditioned stimulus (US; e.g., footshock). Conditioned responses that can be active (rearings, diving, locomotion) or passive (freezing that is cessation of all but respiratory movement) can be used as measures of memory.

Conditioned taste aversion

Aversive learning task in which animals associate a food source (for example, saccharine flavoured water) with malaise usually induced by lithium chloride injection. Avoidance of the food previously associated with malaise is used as a memory index.

Inhibitory avoidance

The apparatus has metal grids on the floor that can deliver a footshock. One part of the grid is covered to provide a safe platform for animals. During training, animals are placed on the safe platform and once they voluntarily step down to the grids they automatically receive a shock. Measuring the time that animals spend on the platform before stepping down assesses memory.

Passive avoidance

The animal learns to inhibit a natural tendency, namely to step into an apparently safer dark compartment that has previously been associated with footshock.

T-maze

This maze is shaped like the letter T (sometimes could be Y) providing the mice with a straightforward choice. It is used to study different spatial memory parameters such as alternation task, delayed-alternation task, among others. It is often used to study working memory.

Social recognition test

The main idea of this test is the same for the object recognition test. In this case the objects are replaced with animals (juveniles, from different cages, different strains). Mice have to explore more the new animal in the test session to have a higher discrimination index indicating a good social memory.

Box1. **Behavioral tests for learning and memory** (Lee and Silva, 2009).

The paradigm that we have mainly used in this thesis is the object recognition. This test is a behavioral model designed to evaluate recognition memory, for example the ability to judge whether something is novel or familiar. Behavioral tests that evaluate the ability of recognizing a previously presented stimulus constitute the core of animal models of human amnesia (Baxter, 2010). This kind of memory is mainly controlled by the hippocampus, although other brain regions cannot be excluded. The novel object recognition test, which has been used since 1988 (Ennaceur and Delacour, 1988) can be configured to study different memory types such as short-term memory, long-term memory or working memory (Antunes and Biala, 2012, Goulart et al., 2010). This memory relies primarily on a rodent's innate exploratory behavior in the absence of externally applied rules or reinforcement (no external motivation, reward or punishment). It is worthy to mention that novel object recognition test and other alternatives have become useful tools for basic and preclinical research as it allows studying the neural basis of memory (Antunes and Biala, 2012).

The task procedure for the novel object recognition test consists of three phases: habituation, training and test phase. In the habituation phase, the animal is allowed to explore freely the maze without objects. During the training phase, the animal is put back to the maze containing two identical objects for a few minutes. Finally, during the test phase, the animal is returned to the maze with two objects, one is identical as in the training and the other one is completely novel (Ennaceur, 2010). When animals are exposed to a familiar and a novel object, they approach frequently and spend more time exploring the novel than the familiar one (Ennaceur, 2010). The memory

performance can be calculated through different indexes, including discrimination index, index of global habituation, or preference index depending on the aim of the study (Gaskin et al., 2010). Some modifications can be made to the original protocol described above in order to study different memory types (Antunes and Biala, 2012).

It is also important to note that the object recognition task evaluates memory for unique episodes or events (one-trial learning), which makes it more sensitive to amnesic experimental interventions, compared to other paradigms. Thus, it allows studying the effects of a drug, genetic or experimental manipulation on different stages of memory (acquisition, consolidation and retrieval). However, several factors might influence the performance in this task, including mouse strains, age and sex of the animals, the presence of spatial cues, type of the objects, duration of the trials, interval period between training and test as well as the dimensions, shape and illumination of the apparatus in which the test is performed *(Table 9)* (Schimanski and Nguyen, 2004, Antunes and Biala, 2012).

Material	Plywood, plastic, Plexiglas, acrylonitrile butadiene styrene, polyvinyl chloride plastic (PVC), wood.
Shape	Rectangular, quadrangular, circular.
Color	Black, opaque, gray, white, transparent.

Table 9. **Apparatus (material, shape and color) used in the novel object recognition task.**

2.3. Role of the endocannabinoid system in memory

The endocannabinoid system plays an important role in synaptic plasticity. Several years of research have demonstrated that the two main endocannabinoids, anandamide and 2-AG, could play a different role in synaptic regulation (Katona and Freund, 2012) *(Figure 19)*. A form of synaptic plasticity attributed to endocannabinoids was the depolarization-induced suppression of inhibition that was first observed in the hippocampus (Pitler and Alger, 1992, Llano et al., 1991). In addition, it has also been demonstrated that endocannabinoids are released on demand to inhibit GABA transmission at presynaptic sites regulating this depolarization-induced suppression of inhibition (Wilson and Nicoll, 2001, Kano et al., 2009). The discovery of endocannabinoid-mediated depolarization-induced suppression of inhibition was soon followed by the description of depolarization-induced suppression of excitation (Kreitzer and Regehr, 2001) and also by findings that anandamide and/or 2-AG mediated LTD or LTP (Robbe et al., 2002).

Endocannabinoids might play an important role in the early stages of memory acquisition. Anandamide has been often regarded as strong modulator of acquisition phases (Murillo-Rodriguez et al., 1998) and it is apparently involved in memory consolidation and extinction (Luchicchi and Pistis, 2012). The role of 2-AG in the regulation of mnemonic functions has also been investigated. 2-AG plays a prominent role as a retrograde messenger in the hippocampus, where it modulates short- and long-term forms of plasticity. Pharmacological and genetic studies report a controversial role of 2-AG in memory. Indeed, some studies show improved performance with enhanced

levels of 2-AG (Pan et al., 2011), whereas others show cognitive impairment (Vigano et al., 2009). It appears that anandamide and 2-AG have segregated roles in memory and learning regulation. Until now, the role of anandamide in memory has been extensively demonstrated whereas recent evidences show that 2-AG could be also involved in memory processing.

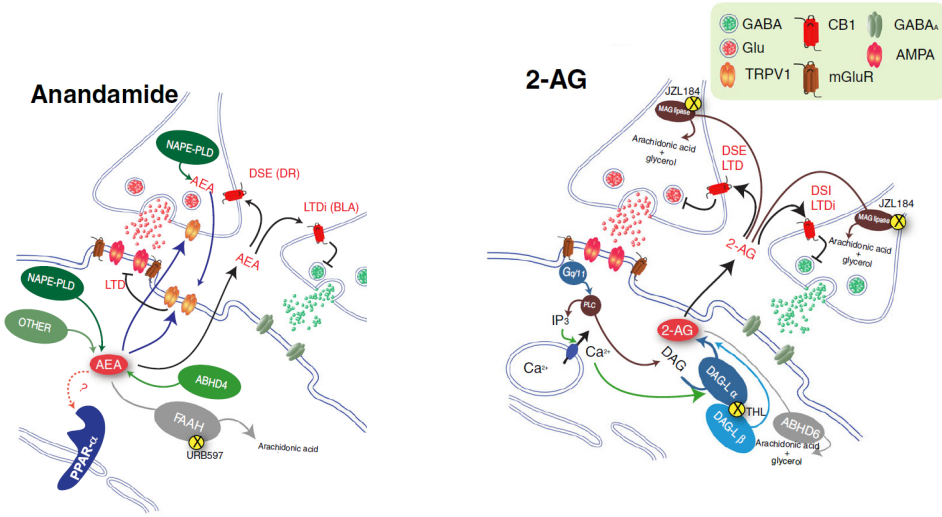


Figure 19. **Synaptic mechanisms of action for anandamide and 2-AG.** Anandamide could activate presynaptic or postsynaptic TRPV1 producing LTD. Anandamide also binds to CB1R producing LTD and depolarization-induced suppression of excitation. On the other hand, 2-AG activates CB1R on axon terminals and inhibits glutamate or GABA release triggering short- and long-term forms of synaptic plasticity *(Pistis et al., 2002).*

2.4. Effects of cannabinoid exposure

Cognitive decline following marijuana consumption has been known since decades in human and similar cognitive impairment has been revealed in laboratory animals. Cannabis use affects cognitive performance, attention, working memory, verbal learning and mental flexibility in humans, among others (Lundqvist, 2005). This impairment is related to the dose and the time of consumption, getting worse with increasing years of regular cannabis use (Solowij et al., 2002).

2.4.1. Human relevance

Prevalence rates for cannabis use have increased in recent years and chronic, heavy cannabis use is a growing health concern. In Spain, the cannabis consumption has a high prevalence in the adolescent population (ESTUDES, 2010). Cannabis use impairs cognitive functions on different levels. Thus, marijuana affects from basic motor coordination to more complex executive function tasks, such as the ability to plan, organize, solve problems, make decisions, remember, and control emotions and behavior. The deficits differ in severity depending on the quantity, age of onset and duration of marijuana use (Crean et al., 2011).

A highly sensitive period for the deleterious effects of cannabis consumption is the adolescence. The brain undergoes protracted development, continuing throughout adolescence and beyond (Giedd et al., 1999). A recent study (Meier et al., 2012) shows that adolescence might represent a period in which brain development is particularly sensitive to environmental input. Thus, persistent cannabis use is

associated with a significant decline in cognitive ability that is significantly greater for people who began using cannabis at an early age. Moreover, if cannabis use starts in adolescence before 18 years, the cognitive deficit remains significant when people has stopped using for at least 1 year before testing. These results provide prospective evidence that adolescent cannabis use is more damaging to cognitive abilities than in adult use (Blakemore, 2012).

2.4.2. Cannabinoid agonists

In animal models, the activation of cannabinoid receptors by endogenous or exogenous cannabinoid agonists impairs learning and memory affecting mainly the hippocampus (Wise et al., 2009). Different hippocampus-dependent memory paradigms, such as T-maze, 8-arm radial maze, spatial alternation or object recognition, are impaired after cannabinoid administration (Davies et al., 2002). The hippocampal affectation by cannabinoids is demonstrated by different studies revealing that cannabinoid agonists infused intrahippocampally induce similar memory deficits to those observed by systemic injection (Lichtman et al., 1995, Suenaga and Ichitani, 2008, Suenaga et al., 2008, Han et al., 2012). Moreover, CB1R blockade reverses those effects revealing a crucial role of these hippocampal cannabinoid receptors (Wise et al., 2009).

Electrophysiological data also demonstrate the crucial role of the hippocampus in the cognitive decline produced by cannabinoids agonists. Thus, it has been shown that the activation of CB1R by exogenous or endogenous agonists depresses hippocampal cell firing (Hampson and Deadwyler, 2000), decreases LTP in the hippocampus

(Hoffman et al., 2007) and reduces the power of hippocampal oscillations and activity (Robbe et al., 2006). Importantly, these electrophysiological results correlate with the memory impairment in behavioral paradigms. Although other brain regions cannot be excluded, we focused our work in the hippocampus.

Some results are contradictory regarding the consequences of chronic cannabinoid exposure. The type of cannabinoid agonist, the dose and the duration of the treatment and the behavioral task have to be considered to understand the discrepancies in these results (Solowij and Battisti, 2008). A body of evidence has demonstrated that the chronic effects are reversible after cannabis cessation (Pope et al., 2002, Grotenhermen, 2007), although the age of cannabis consumption is critical (Schweinsburg et al., 2008, Blakemore, 2012). However, more research is needed in order to confirm this in laboratory animals and in humans. Finally, tolerance development to the cognitive effects induced by cannabinoid agonists is still unclear and it depends on the age of onset, dose, and duration of the treatment (Solowij and Battisti, 2008). New insights in the long-term effects of cannabis will be further explained in the results and discussion sections.

2.4.3. Cannabinoid antagonists

The memory impairment produced by cannabinoid agonists suggests the possibility that blockade of CB1R may lead to an enhancement of certain memory processes. Indeed, rimonabant has been shown to improve certain aspects of memory in rodents, such as a facilitation of olfactory memory (Terranova et al., 1996) and working memory

(Hampson and Deadwyler, 1998). In agreement, mice lacking CB1R show an increase of LTP in the hippocampus (Bohme et al., 2000), an improvement in memory retention in the novel object recognition test (Reibaud et al., 1999, Maccarrone et al., 2002) and an increased number of conditional changes in the active avoidance task (Martin et al., 2002). However, other studies do not reveal any cognitive effect after the pharmacological blockade of CB1R (Lichtman et al., 2002). On the other hand, the CB1R has been related with memory extinction and forgetting processes. Pharmacological and genetic deletion of CB1R produces an impairment of the memory extinction in the fear conditioning test (Lichtman et al., 2002) revealing a crucial role of CB1R in this process (Marsicano et al., 2002).

2.5. Possible mechanisms underlying memory impairment by cannabinoids

The mechanisms involved in the modulation of learning and memory by cannabinoids have been widely investigated. Different neurotransmitter systems that are modulated by the endocannabinoid system are involved in this cognitive impairment. Thus, cannabinoid-induced memory deficits have been related to a decrease in the cholinergic activity in the CNS (Braida and Sala, 2000) or to a suppression of GABA release producing an increase in excitatory firing (Katona and Freund, 2012), among others. In this regard, THC administration decreases GABA levels and increases glutamate levels in the rat prefrontal cortex (Pistis et al., 2002). Interestingly, recent data demonstrate that astrocytic CB1R also participate in the regulation of the working memory impairment induced by cannabinoids (Han et al., 2012).

On the other hand, the activation of cannabinoid receptors leads to the engagement of numerous signal transduction pathways (Bosier et al., 2010b). Our group has previously demonstrated that THC modulates PI3K/Akt pathway and the glycogen synthase kinase-3 in the hippocampus after acute exposure (Ozaita et al., 2007). Moreover, we have proposed a possible mechanism that could explain the acute amnesic-like effects of THC *(Figure 20)*. Thus, an unbalance between glutamatergic and GABA-ergic transmission leads to the activation of mTOR pathway that is associated to the impairing effects of THC in two cognitive tests involving the hippocampus, the novel object recognition test and the context-recognition test (Puighermanal et al., 2009). However, the mechanism that underlies the memory effects of endocannabinoid modulation or the chronic cannabinoid agonist administration remain unclear.

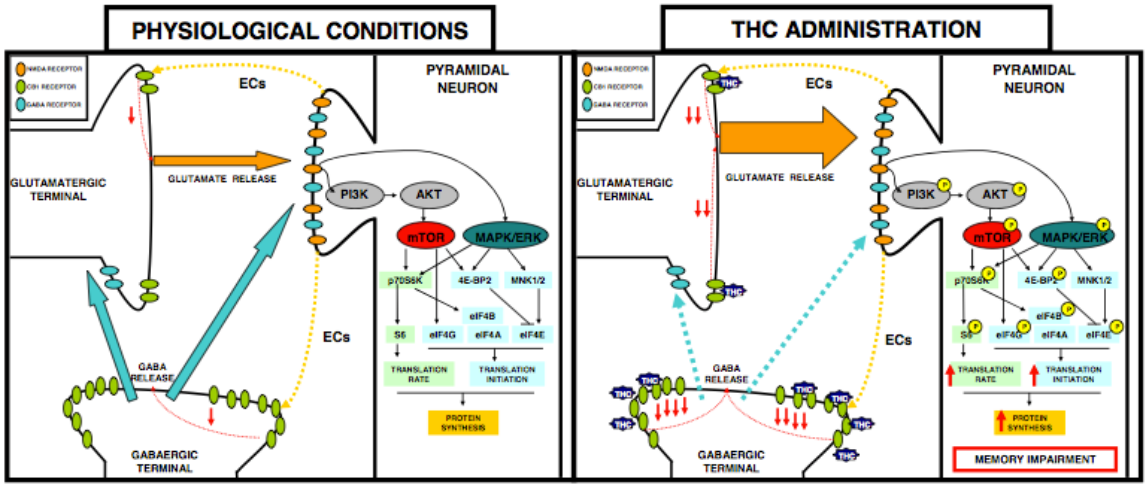


Figure 20. **Schematic representation showing a possible mechanism involved in THC amnesic-like effects.** On the left, the physiological conditions are represented. mTOR pathway is localized in the somatodendritic compartment of the pyramidal neurons whereas CB1R are mainly localized in GABAergic neurons and to a minor extent in glutamatergic neurons. Endocannabinoid system, through CB1R, modulates the neurotransmitter release in GABAergic and glutamatergic terminals. At the postsynaptic level, mTOR and MAPK/ERK pathways are activated by glutamate receptors and modulate protein synthesis by regulating the translation rate and translation initiation. On the right, a possible mechanism to explain the amnesic-like effects caused by THC. An unbalance between GABAergic and glutamatergic neurotransmission is produced by the THC mainly acting on CB1R located in GABAergic neurons. This unbalance leads to a glutamatergic activation of the mTOR pathway resulting in the phosphorylation of different downstream targets such as p70S6K, S6, 4E-BP2, eIF4E, eIF4B and eIF4G. This activation promotes an increase of the translation rate and translation initiation leading to an enhancement of the protein synthesis and the consequent amnesic-like effects promoted by THC *(Puighermanal et al., 2009)*.

3. Fragile X syndrome

3.1. General features of the fragile X syndrome

Fragile X syndrome is the most common form of inherited intellectual disability and the leading monogenic cause of austism spectrum disorders (Kooy et al., 2000). In almost all known cases of fragile X syndrome, the causative mutation is a trinucleotide CGG expansion in the promotor region of the fragile X mental retardation gene (Fmr1) *(Figure 21)*. In humans, the number of CGG repeats is highly polymorphic. When the number of repeats reaches over 200, it leads to hypermethylation and epigenetic silencing of Fmr1. In consequence, the fragile X mental retardation protein (FMRP) is lost and causes the fragile X syndrome (Krueger and Bear, 2011, Penagarikano et al., 2007). This protein is a RNA-binding protein that has a major role in the negative regulation of the translation of bound mRNAs, especially at synapses in neurons. Loss of FMRP impairs normal synaptic plasticity, which is believed to be the molecular basis of intellectual disability in fragile X syndrome patients (Bassell and Warren, 2008).

3.2. Fragile X syndrome phenotypes

The clinical presentation of fragile X syndrome varies considerably. Patients with fragile X syndrome usually exhibit neurodevelopmental problems, including attention deficit, hyperactivity disorder, and autistic-like behavior (Cornish et al., 2008, Hagerman, 2006). Additional features of fragile X syndrome include mild facial

abnormalities, macroorchidism, sleep problems and epileptic seizures (Gould et al., 2000, Merenstein et al., 1996, Berry-Kravis and Potanos, 2004). Several cellular and molecular alterations have also been demonstrated in fragile X syndrome (Wang et al., 2012, Santoro et al., 2012). Most of these phenotypes have been studied in mouse models of fragile X syndrome *(Table 10)*. The main animal model for this disease was obtained by interrupting the murine Fmr1 (Bakker, 1994) (Fmr1KO) that causes the lost of FMRP production mimicking the situation observed in humans. However, other genetic models have been generated *(Table 10)*.

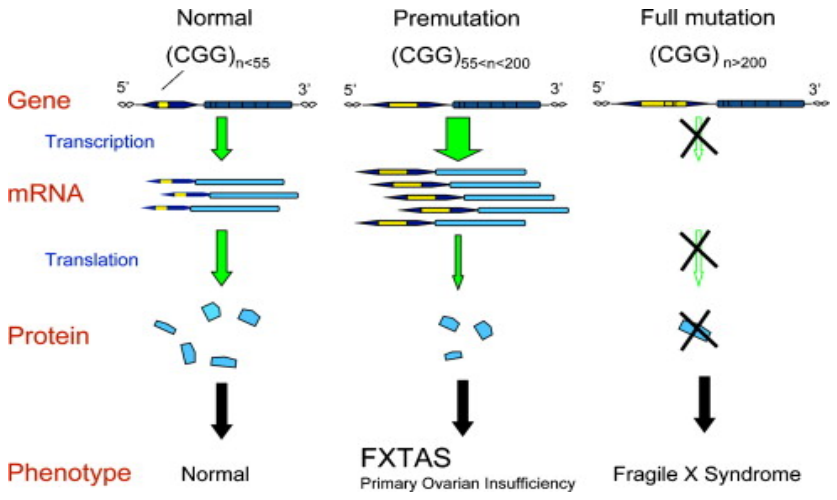


Figure 21. **CGG expansion in the promotor of Fmr1 produces ablation of FMRP producing fragile X syndrome** *(Hagerman and Hagerman, 2002).*

Mouse Model	Genetic approach	Modification	References
Fragile X Knokout mouse	Knockout	Fmr1 KO	(Bakker, 1994)
FXR1 mouse	Paralogus genes	FXR1 KO	H. Siomi (pers. Commun.)
FXR2 mouse	Paralogus genes	FXR2 KO	(Bontekoe et al., 2002)
Transgenics	Repeat expansion	Different CGG copies (43, 60, 97)	(Bontekoe et al., 1997, Lavedan et al., 1997, Lavedan et al., 1998)
Knock-in	Repeat expansion	96 CGG copies	(Bontekoe et al., 2001)
Transgenic	Repeat expansion	Fmr1 cDNA Fmr1 YAC	C.E. Bakker (pers. Commun.) Peier, McIlwain et al. 2000)

Table 10. **Mouse models of the fragile X syndrome.** Different mouse models for the fragile X syndrome have been created using different genetic approaches. However, the Fmr1 KO most characterized is the typical deletion on the Fmr1 gene *(Kooy, 2003).*

3.2.1. Behavioral alterations

The behavioral phenotype has been largely investigated in the Fmr1KO mouse, the most characterized mouse model to study fragile X syndrome. It presents several behavioral alterations including modifications in anxiety-related responses, labile mood, hyperactivity, autistic traits such as deficits in social interactions, reciprocal

communication, cognitive and behavioral inflexibility, decreased pain processing and sensory hypersensitivities leading to an increased susceptibility to audiogenic seizures (Wijetunge et al., 2012, Price et al., 2007).

A prominent behavior found in Fmr1KO is a mild learning deficit in different spatial learning tasks, such as the radial arm maze or the Morris water maze (Mineur et al., 2002, Dobkin et al., 2000, Van Dam et al., 2000). These cognitive deficits could be due to hippocampal defects (Logue et al., 1997). Contradictory results appear in context conditioning test because some groups have found differences between Fmr1KO and WT mice (Paradee et al., 1999), whereas other laboratories do not find differences. This could be due to the different experimental conditions, including the context conditioning protocol, the intensity of the shock or the apparatus used. In addition, Fmr1KO mice show an impaired acquisition of a visuospatial discrimination task as well as a stronger spatial preference (Krueger and Bear, 2011). Overall, it is accepted that fragile X syndrome is accompanied by a cognitive inflexibility and impairment in several memory tasks.

Anxiety is a common symptom associated with fragile X syndrome, although some contradictory results have appeared. One study reveals lower anxiety in the Fmr1KO mice using the open field and light-dark transition test (Peier et al., 2000). In contrast, others do not reveal differences in anxiety levels using the elevated plus maze (Mineur et al., 2002). However, a recent study demonstrates an anxiolytic-like behavior in the Fmr1KO mice (Jung et al., 2012). Importantly, anxiety-like alterations in these mice have been related to social behavioral deficits (Wijetunge et al., 2012).

Another important behavioral feature of fragile X syndrome is the self-injurious behavior (Symons et al., 2003), suggesting alterations in pain processing. Interestingly, FMRP is expressed by nociceptors and localized in pain-sensing neurons as well as in regions implicated in nociception (Price et al., 2006). In this regard, a recent study using behavioral and electrophysiological approaches shows a marked impairment in the plasticity of the nociceptive system with a crucial role of mGluR5 and the mTOR pathway in the Fmr1KO (Price et al., 2007). Self-injurious behaviors may be reduced by increasing pain sensitivity (Symons et al., 2004) and this could be one of the therapeutic goals in the fragile X syndrome. However, the connection between self-injurious behavior and pain sensitivity is not fully understood (Symons and Danov, 2005).

Finally, fragile X syndrome patients have an increased rate of epilepsy and this is reflected in the Fmr1KO mice by an increased susceptibility to audiogenic seizures (Musumeci et al., 2000). Thus, Fmr1KO present an elevated seizure response to intense auditory stimuli compared to WT on different genetic backgrounds (Michalon et al., 2012). This has been one of the most robust and reproducible phenotypes in the Fmr1KO that correlates with human patients.

3.2.2. Cellular and molecular alterations

Dendritic spines have been closely related to synaptic function (Bonhoeffer and Yuste, 2002). Alterations in spine morphology and density are post-mortem features of neurons in patients with intellectual disability (Kaufmann and Moser, 2000). The most common cellular phenotype associated with the loss of FMRP in both

mouse and human is an aberrant increase in the immature dendritic protrusions or filopodia. Interestingly, this immature spine morphology profile is observed in the hippocampus, cerebellum and neocortex of Fmr1KO mice (Wijetunge et al., 2012). However, there are discrepancies that might be due to numerous factors including differences in experimental design, genetic background, brain region, age or cell type examined (He and Portera-Cailliau, 2012). All these findings suggest that FMRP regulates a myriad of cellular processes involved in shaping synapse morphology and density. Recent *in vivo* studies show changes in spine dynamics that offer a new way of understanding the role of FMRP in regulating neuronal development.

It is important to consider the functional significance of altered dendritic spine number and morphology and how they relate to neuronal or circuit function *(Figure 22)* (Portera-Cailliau, 2012). In Fmr1KO mice, the structural abnormalities of synapses are paralleled by significant changes in functional synaptic connectivity (Pfeiffer and Huber, 2009). Several studies have investigated whether the loss of FMRP results in impairments or alterations in synaptic plasticity. The two principal findings observed in the Fmr1KO are an enhanced Gq-coupled receptor-dependent LTD and an impaired cortical LTP (Pfeiffer and Huber, 2009). These observed abnormalities might be related to the cognitive deficits observed in the fragile X syndrome since LTP and LTD can directly influence learning and memory.

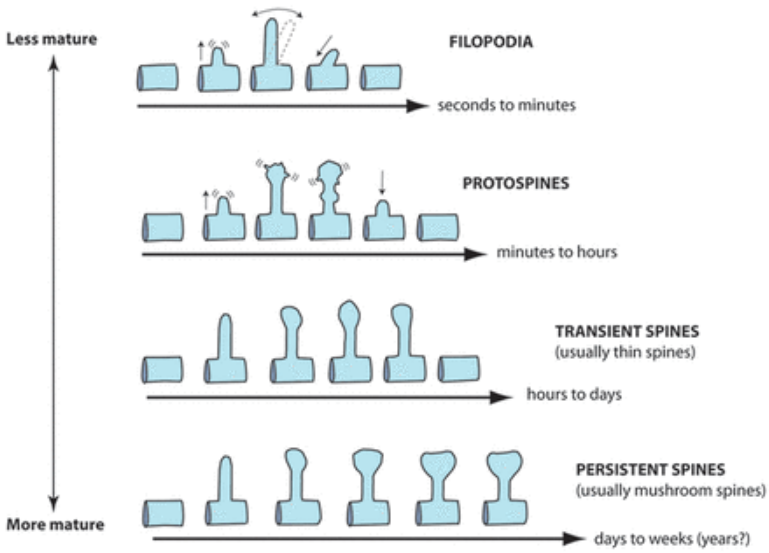


Figure 22. **Proposed classification schemes for dendritic spines based on dynamics and lifetime.** Spines can be distinguished between filopodia (very short-lived protrusions that do not establish functional synapses), protospines (or spine precursors that establish rudimentary synapses early in development), transient spines (a mature protrusion that has a lifetime of less than a few days), and persistent spines (a mature protrusion that makes long-lived and stable synapses that last weeks to months, possibly years). This classification scheme applies to pyramidal neurons in the neocortex and hippocampus. It is inspired by live imaging studies in slices and *in vivo (Portera-Cailliau, 2012).*

In 2004, the mGluR theory *(Figure 23)* was postulated to explain several aspects of the clinical manifestations of fragile X syndrome patients and Fmr1KO mice (Bear et al., 2004). This theory proposed that the high density of spines and immature spines, the electrophysiological deficits in Fmr1KO after activation of mGluR5 and the behavioral phenotypes, are caused by an exaggerated AMPA receptor internalization induced by the overactivation of group I

mGluR (mGluR1 and mGluR5). This model predicts that the increased translation of different mRNAs due to the absence of FMRP perturbs receptor internalization dynamics, thereby exaggerating internalization of AMPA receptors and, consequently, weakening the synapse. In agreement, the mGluR LTD is enhanced in the Fmr1KO (Huber et al., 2002) because the proteins that are important for the maintenance of this LTD are already abundantly present at the synapses (Levenga et al., 2010).

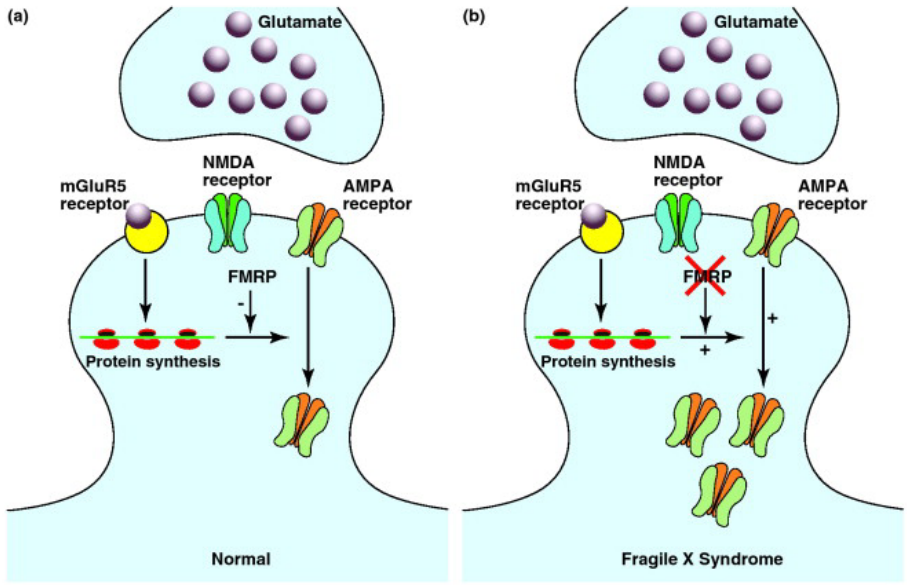


Figure 23. **The mGluR theory of fragile X syndrome.** A) Stimulation of mGluR5 by glutamate induces local mRNA translation at the synapse. Local protein synthesis stimulates the internalization of AMPA receptors, which is essential for long-term synaptic plasticity. FMRP negatively regulates transcription and reduces the internalization of AMPA receptors. B) In Fmr1*KO* mice and in neurons from patients with fragile X syndrome an increased internalization of AMPA receptors is produced due to the absence of FMRP, which weakens the synapse *(Levenga et al., 2010).*

Another accepted theory to understand the fragile X syndrome pathophysiology is an altered GABA receptor signaling (D'Hulst and Kooy, 2007). GABA, through $GABA_A$ and $GABA_B$ receptors, is the major inhibitory neurotransmitter in the CNS. Interestingly, mRNAs encoding $GABA_A$ receptors subunits are targets of FMRP (Miyashiro et al., 2003). Different studies have reported decreased mRNA and protein levels of several $GABA_A$ subunits (Curia et al., 2009, D'Hulst et al., 2009) or changes in expression of the GABA synthesizing enzyme glutamate decarboxylase (El Idrissi et al., 2005). This altered GABA signaling in the Fmr1KO mice results in decreased GABA receptor signaling efficiency in the hippocampus, down-regulation of tonic GABA receptor inhibition and morphological defects of GABA releasing interneurons in some brain regions compared to WT (Levenga et al., 2010). Recently, $GABA_B$ deficits are also linked to fragile X syndrome (Pacey et al., 2009). Overall, these two theories suggest an exaggerate excitatory mGluR signaling and a decreased GABA signaling, suggesting an excitatory/inhibitory unbalance that could explain most of the fragile X syndrome traits.

It has been also suggested that FMRP could potentially regulate translation at the presynaptic compartment in an activity dependent manner (Akins et al., 2009). It is an intriguing possibility that the cognitive and behavioral deficits apparent in autistic patients could arise in part from alterations of local translation in the presynaptic compartment. Perturbations in this process could result in abnormal experience-dependent synaptic plasticity – a leading hypothesis for the neurobiological cause of autism (Akins et al., 2009). Moreover, other molecular components altered in the fragile X syndrome, including the

endocannabinoid system and the mTOR pathway will be described in the next sections.

3.3. Therapeutic targets identified in different mouse models to treat fragile X syndrome

To date, the treatment of patients with fragile X syndrome is symptomatic. Psychostimulants treatment ameliorates attention deficit and hyperactivity and selective serotonin reuptake inhibitors can reduce aggression and anxiety-like responses. Moreover, it has been demonstrated in animal models that an enriched environment can improve behavior in a fragile X syndrome model (Restivo et al., 2005). Recently, new strategies for therapeutic intervention have been proposed based on the mGluR and GABA theories *(Figure 24)*. In addition, recent data using genetic and pharmacological approaches have identified novel promising targets to treat fragile X syndrome.

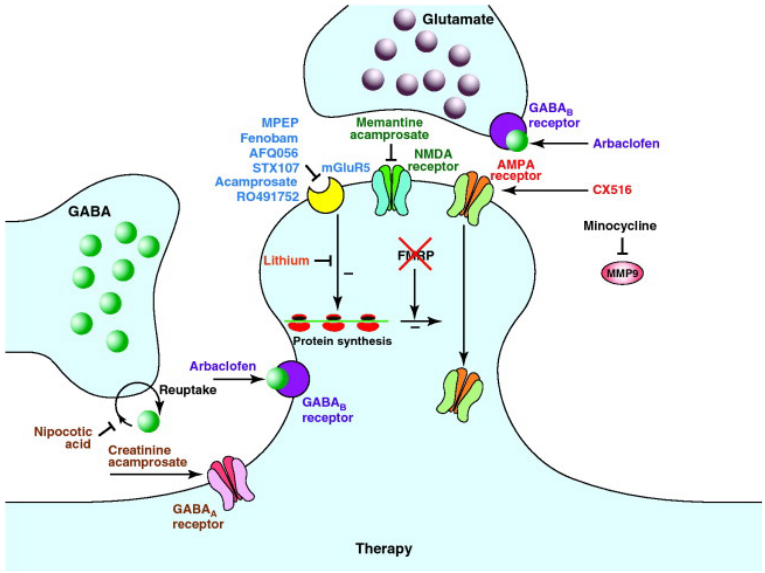


Figure 24. **Therapeutic strategies for fragile X syndrome.** Drugs can interact with different types of neuronal receptors, which might rescue the disturbed synaptic transmissions in fragile X syndrome. Negative mGluR5 modulators, $GABA_A$ receptor agonists, $GABA_B$ receptor agonists, NMDA receptor antagonists, positive AMPA receptor modulators are potential candidates to rescue the fragile X syndrome phenotype by correcting altered synaptic plasticity. In addition, other therapeutic interventions, such as lithium, minocycline and acamprosate, also have a therapeutic rationale and might have beneficial effects on behavioral and cognitive phenotypes of fragile X syndrome *(Levenga et al., 2010)*.

3.4. mTOR pathway in the fragile X syndrome

Components of the mTOR signaling cascade are present at synapses and influence synaptic plasticity via regulation of local protein synthesis (Tang and Schuman 2002). mTOR is activated in dendrites by stimulation of group I mGluRs and is required for mGluR-LTD at CA1 synapses (Hou and Klann 2004). Growing evidence indicates that

deregulation of mTOR is associated with several human diseases, including cancer, diabetes, autism and other neuronal disorders (Sabatini, 2006, Troca-Marin et al., 2012, Dann et al., 2007). In this line, several groups have recently analysed the Akt/mTOR pathway in Fmr1KO mice *(Figure 25)* (Gross et al., 2010, Osterweil et al., 2010, Sharma et al., 2010a). Indeed, increased activities of PI3K, Akt, and mTOR have been detected in cortical synaptoneurosomes and hippocampal lysates from Fmr1KO mice (Sharma et al., 2010a, Gross et al., 2010). Additionally, the inhibition of PI3K specifically rescues the excess translation and subsequent AMPA receptor endocytosis revealed in these mutant mice (Gross et al., 2010). In contrast, one study fails to observe increased levels of mTOR pathway components in Fmr1KO mice (Osterweil et al., 2010). Importantly, genetic reduction of an mTOR pathway component, S6K1, prevents in Fmr1KO mice the elevated phosphorylation of translational control molecules, the exaggerated protein synthesis, the enhanced mGluR-LTD, the macro-orchidism, the immature dendritic spine morphology and multiple behavioral phenotypes, including social interaction deficits, impaired novel object recognition test and behavioral inflexibility (Bhattacharya et al., 2012). Overall, the deregulation of mTOR signaling and aberrant mTOR-dependent protein translation has been demonstrated to contribute to different traits of the fragile X syndrome. However, further research will be required in order to develop pharmacological tools against mTOR pathway with a effective therapeutic profit in fragile X syndrome.

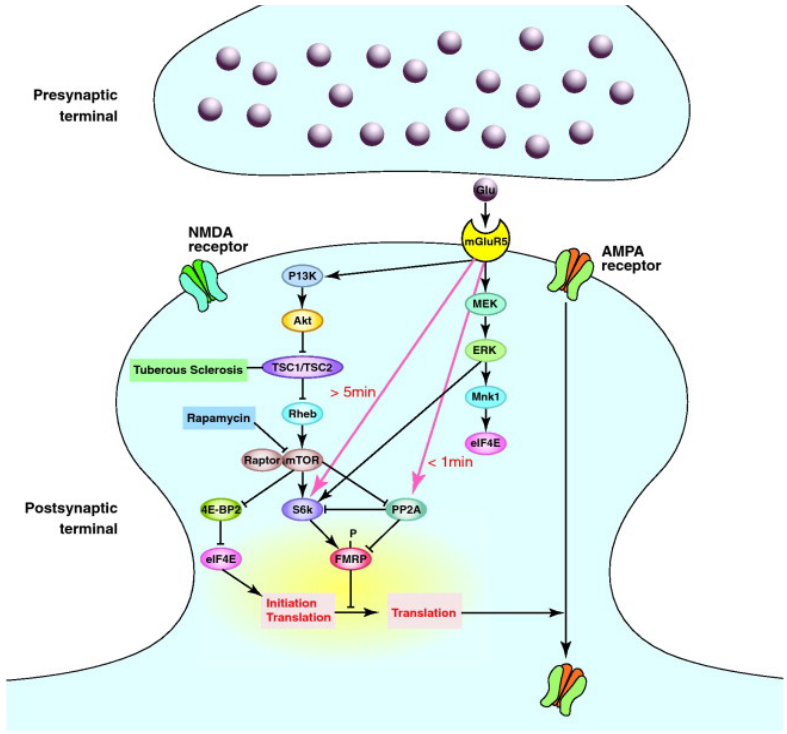
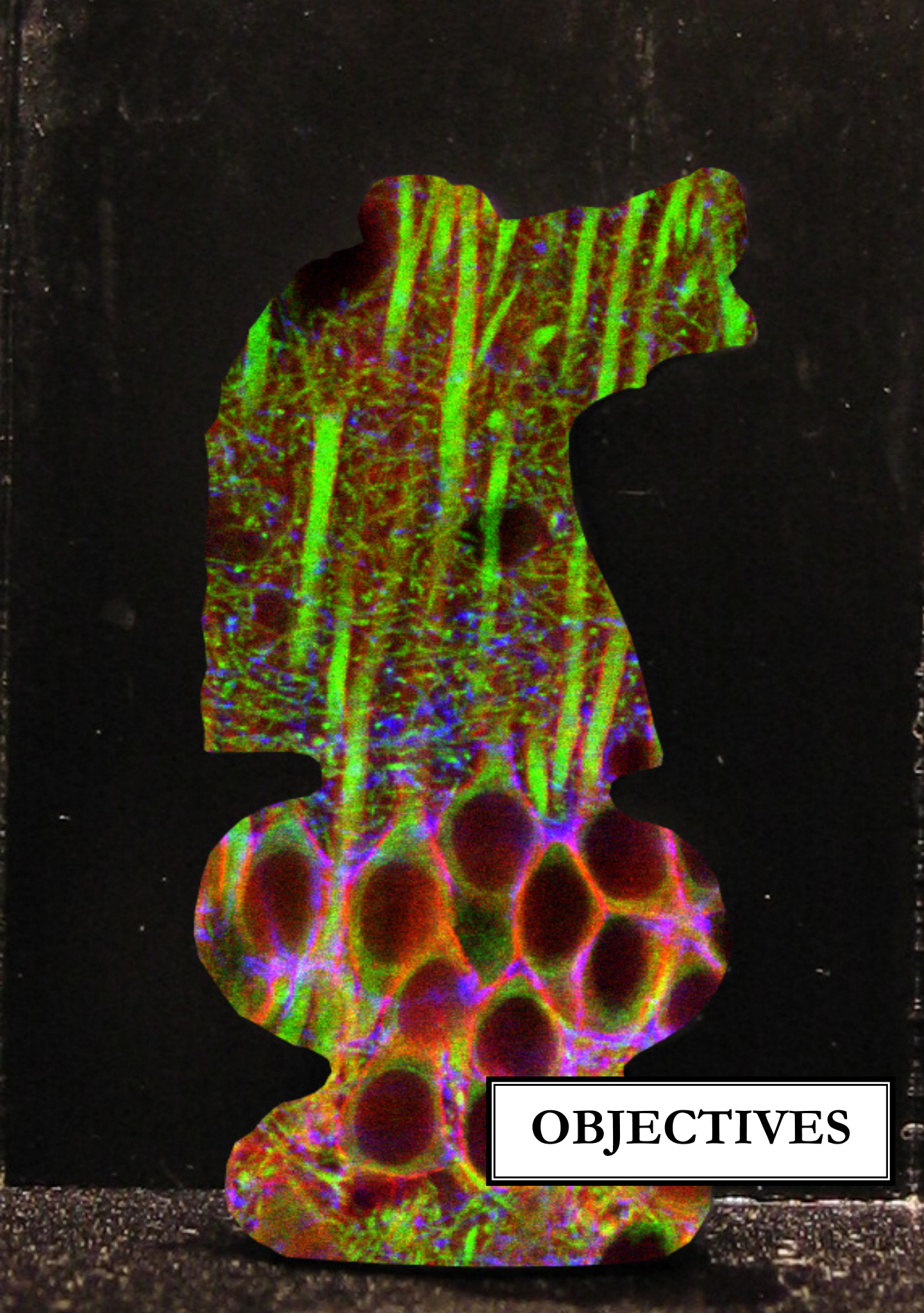


Figure 25. **The mTOR signaling pathway in fragile X syndrome.** An overactivation of mTOR has been shown in fragile X syndrome. Active mTOR can phosphorylate S6K and 4E-BP affecting the translation process. In physiological conditions FMRP represses the translation of target mRNAs. In fragile X syndrome, the overactivation of mTOR pathway and the lack of FMRP promote an exaggerated protein synthesis *(Levenga et al., 2010)*.

3.5. The endocannabinoid system in the fragile X syndrome

Different behavioral and biochemical responses controlled by the endocannabinoid system are affected in the fragile X syndrome, including cognition, anxiety, neuronal excitability and nociception (Kano et al., 2009), as well as the Akt/mTOR signaling pathway

(Ozaita et al., 2007, Puighermanal et al., 2009). In addition, postsynaptic activation of mGluR5 is a key physiological mechanism that promotes the synthesis of endocannabinoids in response to synaptic activity (Varma et al., 2001), thereby triggering eCB-LTD of excitatory transmission (Kano et al., 2009). Importantly, several studies have shown alterations in glutamatergic and GABA-ergic transmission in fragile X syndrome (Centonze et al., 2008, Olmos-Serrano et al., 2010). It has been recently shown that the loss of FMRP results in aberrant mGluR5 signaling pathways and deregulated mGluR5- driven eCB-LTD in several brain areas of adult Fmr1KO (Jung et al., 2012, Zhang and Alger, 2010). These findings point to the possibility that defective endocannabinoid modulation of synaptic function may contribute to different traits of the fragile X syndrome. Instead, a recent study demonstrated that the macromolecular complex that links mGluR5-dependent 2-AG formation is altered in the glutamatergic synapses of Fmr1KO mice (Jung et al., 2012). However, more studies should be done to clarify the role of the endocannabinoid system in the pathophysiology of fragile X syndrome in order to facilitate the development of novel therapeutic strategies.


OBJECTIVES

Objective 1

To set up and validate an improved object recognition task protocol in order to study memory in mice.

**Main articles #1, #2 and #3 and article #1 from the annex present results using this improved novel object recognition test.*

Objective 2

To dissect the functional role of AEA and 2-AG in cognitive performance, nociception and anxiety-like behavior using acute and chronic pharmacological approaches to specifically enhance their endogenous levels in different behavioral tasks.

Article #1:

Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses.

Arnau Busquets-Garcia*, Emma Puighermanal*, Antoni Pastor, Rafael de la Torre, Rafael Maldonado, Andrés Ozaita.

Biol Psychiatry. **70**:479-86 (2011)

Objective 3

To study the relevance of the mTOR signaling in the behavioral and molecular effects of acute and chronic administration of THC.

Article #2:

Dissociation of the pharmacological effects of THC by mTOR blockade.

Emma Puighermanal*, Arnau Busquets-Garcia*, Maria Gomis-González, Giovanni Marsicano, Rafael Maldonado, Andrés Ozaita.

Neuropsychopharmacology (2013)

- In the articles #1 and #3 from the annex we also studied the memory and motor coordination impairment produced by THC administration.

Objective 4

To investigate the involvement of the endocannabinoid system in the behavioral, cellular and molecular traits associated to the fragile X syndrome.

Article #3:

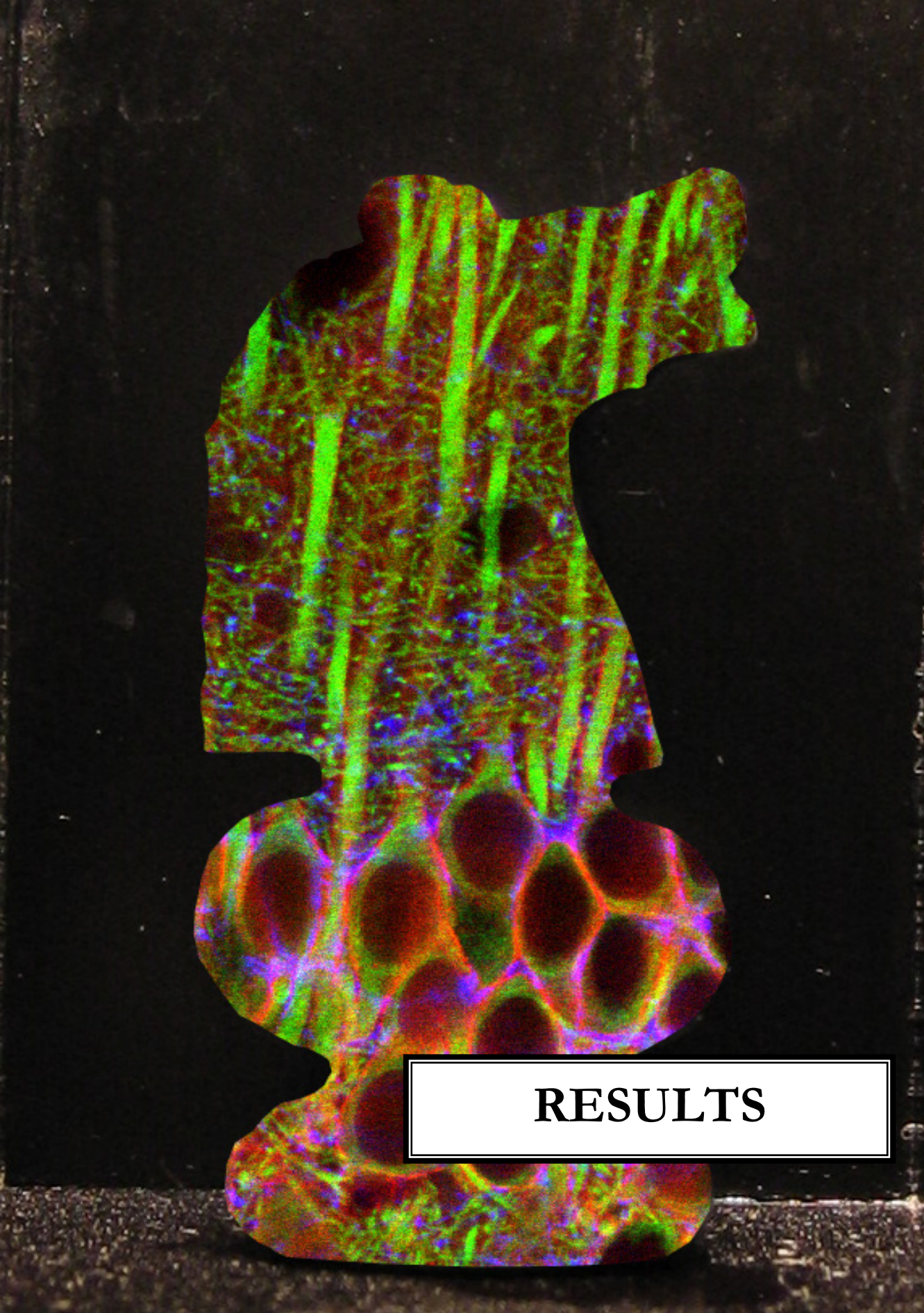
Targeting the endocannabinoid system in the treatment of fragile X syndrome.

Arnau Busquets-Garcia, Maria Gomis-González, Thomas Guegan, Carmen Agustín-Pavón, Antoni Pastor, Susana Mato, Alberto Pérez-Samartín, Carlos Matute, Rafael de la Torre, Mara Dierssen, Rafael Maldonado, Andrés Ozaita.

Nat Med. (2013)

- Main article #4 and article #2 from the annex are reviews related to our work.

- In the Annex there is a "MoU and Patents" section.



RESULTS

ARTICLE 1

Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses.

Arnau Busquets-Garcia*, Emma Puighermanal*, Antoni Pastor, Rafael de la Torre, Rafael Maldonado, Andrés Ozaita.

Biol Psychiatry. **70**(5):479-86 (2011)

*Equal contribution

This article has been presented also in the thesis of Emma Puighermanal

Busquets-Garcia A, Puighermanal E, Pastor A, de la Torre R, Maldonado R, Ozaita A. Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses. Supplemental information. Biol Psychiatry. 2011 Sep 1;70 (5):479-486.

ARTICLE 2

Dissociation of the Pharmacological Effects of THC by mTOR Blockade.

Emma Puighermanal*, Arnau Busquets-Garcia*, Maria Gomis-González, Giovanni Marsicano, Rafael Maldonado, Andrés Ozaita.

Neuropsychopharmacology. 2013; 38(7):1334-1343.

*Equal contribution

Puighermanal E, Busquets-Garcia A, Gomis-Gonzalez M, Marsicano G, Maldonado R, Ozaita A. Dissociation of the pharmacological effects of THC by mTOR blockade. Supplementary figures. Neuropsychopharmacology. 2013 Jun; 38(7):1334-1343.

ARTICLE 3

Targeting the endocannabinoid system in the treatment of fragile X syndrome.

Arnau Busquets-Garcia, Maria Gomis-González, Thomas Guegan, Carmen Agustín-Pavón, Antoni Pastor, Susana Mato, Alberto Pérez-Samartín, Carlos Matute, Rafael de la Torre, Mara Dierssen, Rafael Maldonado, Andrés Ozaita.

Nat Med. 213; 19(5):603-607.

Busquets-Garcia A, Gomis-Gonzalez M, Guegan T, Agustin-Pavon C, Pastor A, Mato S, et al. Targeting the endocannabinoid system in the treatment of fragile X syndrome. Supplementary material. Nat Med. 2013 May;19(5):603-607.

ARTICLE 4

Differential mechanisms involved in cannabinoid responses: possible therapeutic implications

Arnau Busquets-Garcia, Emma Puighermanal, Rafael Maldonado, Andrés Ozaita

Biol Psychiatry *(Under revision)*

Differential mechanisms involved in cannabinoid responses: possible therapeutic implications

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Keywords: endocannabinoid system, cannabinoid receptor, CB1, CB2, monoacylglycerol lipase, anandamide, 2-arachidonoylglycerol, memory, anxiety, nociception, food intake.

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Number of words in the text: 3741
Number of tables: 0
Number of figures: 0
Number of supplementary material:0

Abstract

The endocannabinoid system has raised increasing attention due to the large variety of potential therapeutic applications derived from its physiological role. This system is composed by G-protein coupled receptors with cell-specific expression, the endocannabinoids, their endogenous ligands, and the enzymatic machinery for the synthesis and degradation of these ligands. The receptors and the catalytic enzymes for the endocannabinoids have been targeted with selective drugs that have allowed characterizing specific outcomes of the modulation of the endocannabinoid activity. This information, together with the data related to the intracellular signaling mechanisms associated to the endocannabinoid system, lead to better understanding the potential therapeutic applications. These include the modulation of anxiety, pain and food intake control, and the preclusion of specific side effects such as memory impairment or tolerance. The identification of the specific differential role of each component of the endocannabinoid system in the pharmacological responses related to the therapeutic and side effects of cannabinoids has opened new perspectives to obtain selective therapeutic effects acting on particular endocannabinoid targets.

1. Endocannabinoid system

The derivatives of Cannabis sativa have been used for thousands of years for recreational and medicinal purposes. These preparations were notorious through history for their mind-altering and curative properties. Two decades after the identification of the principal psychoactive ingredient in these preparations, delta9-tetrahydrocannabinol (THC)[1], the main cannabinoid receptor in the brain, CB1 receptor (CB1R) was cloned[2]. A second cannabinoid receptor, the CB2 receptor (CB2R)[3] was also cloned. Both CB1R and CB2R belong to the G protein-coupled receptor superfamily. More recently, the G protein-coupled receptor 55 (GPR55) has been considered as a putative cannabinoid receptor considering its high affinity for cannabinoid agonists[4, 5]. Two main endogenous ligands, so-called endocannabinoids, were characterized: 2-arachidonoyl ethanolamine (anandamide)[6] and 2-arachidonoyl glycerol (2-AG)[7]. Anandamide is a partial CB1R agonist and weak CB2R agonist, while 2-AG, is a full agonist on both CB1R and CB2R with lower affinity and produced in greater amounts than anandamide[8, 9]. Anandamide and 2-AG are synthesized on-demand by N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) and diacyl glycerol lipase (DAGL)[10], respectively. These endocannabinoids are inactivated by different enzymes. Anandamide is degraded by the fatty acid amide hydrolase (FAAH) while 2-AG is mainly metabolized by the monoacylglycerol lipase (MAGL)[9, 10].

a. Anatomical localization

The endocannabinoid system is broadly expressed through the organism[11] contributing to the homeostasic control of different physiological functions[12]. We will focus this review on the specific role of the endocannabinoid system in the central

nervous system (CNS), as they have raised much interest for the potential therapeutic approaches derived from recent research.

CB1R is widely distributed in the CNS, with a strong expression in hippocampus, cerebellum, cortex and striatum, and with reduced expression in thalamus, mesencephalon and spinal cord[13]. These receptors are mainly confined at the presynaptic site on synaptic contacts[8] with a higher expression in GABAergic terminals than in glutamatergic terminals[14]. CB1R are generally coupled to Gi/o inhibitory proteins[15], and its activation prevents neurotransmitter release through the modulation of Ca2+ and K+ presynaptic conductances[16]. These presynaptic CB1R are activated on the bases of enhanced postsynaptic activity, perceived by the synthesis and retrograde release of endocannabinoids[8]. CB1R are also expressed in other brain cell types, such as astroglia[17, 18], where might play important roles modulating glutamate release from the astroglial cells. The development of genetically modified mice lacking CB1R in specific brain cell populations has allowed addressing the significance of CB1R expression and modulation in forebrain glutamatergic or GABAergic neurons for specific behavioral outcomes or responses to agonist treatment[19-21]. These data have allowed focusing the research on those specific targets that may be of particular interest for beneficial or deleterious properties of cannabinoids.

The central localization of CB2R has been largely debated. CB2R expression in neurons seems limited in the healthy brain[3], although, its inducible expression has been described on microglial cells[22] after brain insult, and astrocytes[23].

The enzymes involved in the synthesis and degradation of the endocannabinoids are located in the proximity of the cannabinoid receptors, thereby modulating their function. Importantly, MAGL is present on presynaptic terminals[10], whereas FAAH is mainly found postsynaptically[24]. These enzymes are the targets of specific pharmacological

inhibitors that enhance the tone of endocannabinoids and thereby act as indirect agonists of the cannabinoid receptors[25, 26].

b. Pharmacological targets in the CNS with therapeutic interest

The endocannabinoid system has gained interest as a therapeutic target due to its physiological role in the control of multiple responses depending on the CNS and peripheral organs[27]. At the central level, the endocannabinoid system modulates nociception, anxiety, food intake and cognition, among other responses with therapeutic interest. Most of these responses have been classically related to the activation of CB1R. However, CB2R ligands have gained attention since they do not display some adverse effects related to CB1R activation in the CNS[27]. In addition, the heteromerization of CB1R with other G-protein-coupled receptors in specific brain regions and cellular populations[5] increases the possibility to target pharmacologically particular dimers or heterodimers.

i) Direct agonists

Different licensed medicines that activate directly the cannabinoid receptors have already been developed[27]. THC, also named Dronabinol or Marinol, and its synthetic analogue, Nabilone, have been used for suppressing nausea and vomiting during chemotherapy or as appetite stimulants in patients with AIDS[27]. In addition, Sativex, a 1:1 mixture of THC and cannabidiol, is already accepted in many countries for spasticity relief in multiple sclerosis[27]. The main caveat to these cannabinoid agonists is the appearance of unwanted side effects that include cognitive and psychomotor impairment, disordered temporal perception and euphoria[28]. They may also enhance the incidence of psychosis, panic and anxiety-like responses[29].

CB2R selective agonists may have a potential interest in different pain[27], autoimmune[30] and anxiety-related disorders[31]. Although the therapeutic potential of these agonists have to be further investigated, CB2R agonists seem to avoid the side effects previously described related to CB1R activation. This approach could optimize the beneficial effects and reduce the classical cannabinoid central side effects.

ii) Indirect agonists

Unlike exogenously administered cannabinoid agonists, endocannabinoids may act in a site- and time-specific manner minimizing side effects. Thus, an alternative approach to enhance the activity of the endocannabinoid system has been obtained by developing inhibitors of the metabolizing enzymes for the main endocannabinoids, FAAH and MAGL. These inhibitors produce cannabimimetic responses with potential therapeutic applications in anxiety, pain or addiction control[32-34]. Notably, some of the adverse effects of cannabinoid agonists such as the development of tolerance or the cognitive deficits can be potentially prevented by using these specific inhibitors[31]. In this regard, preclinical studies with different FAAH inhibitors show potential therapeutic uses in analgesia, depression, anxiety, and emesis, among others. In addition, MAGL inhibitors show analgesic and anxiolytic properties[31, 33]. Moreover, the pharmacological enhancement of 2-AG by MAGL inhibition normalized the endocannabinoid-dependent long-term depression at excitatory synapses in the ventral striatum and prefrontal cortex, which is mediated by 2-AG, and these effects correlated with the normalization of the reduced-anxiety behavior in the mouse model[35].

iii) Antagonists/inverse agonists

Antagonists for the CB1R have been an important approach[36] in the treatment of obesity and other related diseases such as cardiometabolic diseases and other disorders associated to an enhanced cannabinoid signaling. Thus, cannabinoid receptor antagonists were initially proposed as a therapeutic tool for obesity and related metabolic disorders[37-39]. The recent involvement of a central overactive endocannabinoid system in specific neuronal disorders, such as Alzheimer disease[40] or schizophrenia[41], suggests that more research is needed to better target the endocannabinoid system inhibition for therapeutic purposes.

Rimonabant, a CB1R antagonist/inverse agonist, was approved as an anti-obesity compound with clinical effectiveness[42]. Unfortunately, rimonabant was withdrawn from the market due to the incidence of central side effects including depression, anxiety and suicidal ideation[43]. However, rimonabant initial success led to the development of comparable drugs, such as taranabant, otenabant or surinabant that showed also the same limitations due to the presence of central side effects[36]. Two novel approaches have raised interest to avoid these central side effects: the development of peripherally acting CB1R antagonists to circumvent the central effects demonstrated by rimonabant, and the development of CB1R neutral antagonists in an attempt to avoid the inverse agonist properties of previous compounds[36].

Antagonists for CB2R have been used mainly at the experimental level for the dissociation of the effects produced by cannabinoid receptor agonists. In this regard, antagonists for CB2R were able to prevent the anxiolytic effect of an enhanced tone of 2-AG[31]. Interestingly, the modulation of the endocannabinoid system could prevent specific traits on a mouse model of fragile X syndrome[44], the most common form of inherited intellectual disability. In this regard, CB1R blockade prevented the memory deficit in the Fmr1 knockout mouse and also improved the traits on nociceptive desensitization, seizure susceptibility, and normalized the synaptic plasticity changes of hippocampal dendritic spines and the mammalian target of rapamycin (mTOR)-

dependent signaling in this brain area[44]. In the same mouse model, CB2R blockade diminished the characteristic reduced non-social anxiety phenotype and the seizure susceptibility[44], pointing to the endocannabinoid system as a suitable therapeutic target for this genetic disease.

Another novel approach to target the endocannabinoid system came from the development of potent and selective inhibitors of the biosynthesis of 2-AG via DAGL, opening novel therapeutic perspectives on diseases that might benefit from a reduction of endocannabinoid tone, such as hyperphagia in obese subjects[45].

2. Cellular and molecular mechanisms involved in cannabinoid central effects related to the therapeutic properties

The last advances in the peculiarities of the endocannabinoid system have provided important insights for better understand the therapeutic actions of cannabinoids in the CNS.

a. Nociceptive modulation

The endocannabinoid system is present in the main spinal and supraspinal structures involved in nociceptive control[46, 47], which results in potent analgesic effects of cannabinoid agonists in multiple animal models and humans[48]. In addition, peripheral cannabinoid antinociceptive actions have also been demonstrated in numerous animal pain models[48]. Noxious stimuli enhance endocannabinoid levels in different nociceptive structures[49, 50], revealing the important physiological role of the endocannabinoid system in the control of pain. CB1R play a crucial role in these responses, although CB2R also participate in central pain modulation, especially in the

central immune responses leading to neuropathic and osteoarthritic pain[51, 52]. Both endocannabinoids, anandamide and 2-AG, induce antinociceptive effects in multiple acute and chronic pain animal models[46, 49, 50]. However, anandamide and 2-AG may have distinct physiological role in the different brain areas involved in the control of pain[53].

The physiological role of endogenous anandamide has been revealed using mice lacking the FAAH enzyme that display enhanced levels of anandamide[54-56] together with an analgesic and anti-inflamatory phenotype[57-59]. Both CB1R and CB2R are responsible for the analgesic effects observed in FAAH-KO, whereas the anti-inflammatory responses would depend on CB2R. On the other hand, enhanced levels of 2-AG due to the removal of MAGL (MAGL-KO) produced a desensitization of CB1R reducing the analgesic responses to exogenous cannabinoid agonists, which difficults the use of these knockout mice to investigate the physiological role of 2-AG[60]. This tolerance seems related to the circulating levels of 2-AG since high doses of the MAGL inhibitor JZL184 produced a similar CB1R de-sensitization[60].

In contrast, low doses of this inhibitor did not develop antinociceptive tolerance[31, 58], suggesting that an experimental approach that would allow a moderate enhancement of 2-AG levels would be required to evaluate the physiological role of this endocannabinoid in order to avoid these adaptive changes. The cellular mechanisms involved remain to be clarified, and more research will be needed to determine the type of endocannabinoid to target depending on the nociceptive stimulus to treat.

b. Anxiety modulation

Cannabinoid agonists modify anxiety-like behavior in animal models and humans[61] producing different effects that depend on the dosage, genetic background and environmental context. Most of the studies have reported biphasic effects of

cannabinoid agonists with anxiolytic-like responses at low doses, whereas higher doses induce anxiogenic-like effects[62]. The mechanisms involved in these biphasic effects on anxiety-related behavior were recently investigated in conditional knockout mice lacking CB1R in specific cell populations. CB1R located in cortical glutamatergic terminals were crucial for the anxiolytic-like effects of low doses of the cannabinoid agonist CP-55,940 whereas CB1R on GABAergic terminals were required to induce the anxiogenic-like responses[21]. Therefore, low doses of cannabinoids would first activate the CB1R on glutamatergic neurons reducing glutamatergic transmission, leading to an anxiolytic-like effect. A positive allosteric modulator of GABA, GS-39783, counteracted the anxiogenic-like effects of CP-55,940 at a high dose[21], suggesting that a reduction of GABA release could indirectly increase the glutamatergic tone explaining the anxiogenic-like responses. This unbalance between excitatory/inhibitory inputs would lead to a differential activation of postsynaptic signaling pathways. Indeed, a THC anxiogenic dose enhanced the phosphorylation of p70S6 kinase (p70S6K), an effector of mTOR, in mouse brain samples, whereas an anxiolytic dose did not affect the activity of this kinase[63]. Notably, only the anxiogenic-like responses triggered by THC were sensitive to mTOR pharmacological inhibition pointing to different molecular mechanisms involved in this biphasic effect of cannabinoids[21].

A role for CB2R in anxiety-related behavior has also been proposed based on the decreased anxiety vulnerability revealed in transgenic mice over-expressing CB2R[64]. In agreement, JWH-133, a selective CB2R agonist, produced dose-dependent anxiolytic-like effects in mice[31], pointing to a dual action of cannabinoid receptors at the control of this specific behavioral effect.

Endogenous anandamide seems to participate in the physiological control of the behavioral responses since the pharmacological or genetic inhibition of FAAH produces anxiolytic-like responses in various animal models through the activation of CB1R[31]. However, FAAH inhibition may lead to unspecific activation of other targets

by the increased levels of several fatty acid amides different from anandamide[65, 66], such as N-palmitoyl-ethanolamine and N-oleoyl ethanolamine[67], that could also produce anxiety-like effects. In addition, the transient receptor potential cation channel subfamily V member 1 (TRPV1) was recently involved in anxiety-like behaviors induced by anandamide[68-70]. Thus, the dual blockade of FAAH and TRPV1 produce more efficacious anxiolytic-like effects in rodents than the selective FAAH inhibition[71]. Anxiogenic situations lead to anandamide release in the amygdala in rodents[72] and increase anandamide serum concentrations in humans. The human anxiety ratings were negatively correlated with anandamide concentrations[73] suggesting that the enhancement of anandamide levels would represent a physiological mechanism to counteract the behavioral manifestations of anxiety under these stressful situations.

2-AG seems to play a similar role to that of anandamide in the control of these behavioral responses since the enhancement of 2-AG levels by inhibiting MAGL enzyme also reduces anxiety-like behavior in different paradigms[11, 74]. Interestingly, the anxiolytic-like effects produced by enhanced levels of 2-AG with low doses of JZL184 were sensitive to pretreatment with the selective CB2R antagonists SR144528 or AM630, and were not observed in CB2R-KO mice[31]. In contrast, the anxiolytic-like effects of high doses of JZL184 under conditions of high environmental averseness or in the marble burying assay were prevented by the CB1R blockade with rimonabant[57, 74]. The use of a different dose of JZL184 could explain these discrepant results since high JZL184 doses or chronic treatments could also interact with FAAH and increase anandamide levels[60, 75].

c. Food intake

The endocannabinoid system plays a crucial role in the control of eating behavior[76] and the pharmacological stimulation of CB1R by exogenous or endogenous

cannabinoids enhances food intake [77-79]. Accordingly, pharmacological or genetic blockade of CB1R reduces food intake[80], and this pharmacological strategy has been proposed for the treatment of obesity and related metabolic diseases[81, 82]. In contrast, CB2R-deficient mice showed increased food intake, and did not develop insulin resistance[83], which suggests a different role of these two cannabinoid receptors in the control of eating behavior. The possible potential interest of CB2R as a target for obesity and metabolic disorders must be clarified with further studies in the near future.

The endocannabinoid system modulates eating behavior in the CNS at two different levels. First, endocannabinoids are released on demand in the hypothalamus to regulate the levels and/or activity of other orexigenic and anorectic mediators such as leptin, ghrelin and melanocortins[76]. Second, the activation of the endocannabinoid system in the mesolimbic dopaminergic pathway enhances the motivation to seek and consume food. Indeed, CB1R are abundant in the brain reward circuit and endocannabinoids are progressively increased in the limbic system following food deprivation[77]. The orexigenic effect of endocannabinoids depends on CB1R located at the terminals of cortical glutamatergic neurons, whereas CB1R signaling on GABAergic neurons suppresses food intake[84]. CB1R signaling in the periphery is also involved in the metabolism and storage of nutrients[81, 82] and could participate in the control of central orexigenic and anorexigenic signals[85]. CB1R in the sympathetic system also modulate lipolysis and fatty acid oxidation[86]. Accordingly, conditional mutant mice lacking CB1R in the forebrain and sympathetic neurons are resistant to diet-induced obesity due to increased lipid oxidation and thermogenesis that result from an enhanced sympathetic tone[86].

Both anandamide and 2-AG have been reported to participate in food intake control, although the specific role of each endocannabinoid has not been yet clarified. Interestingly, mice over-expressing MAGL specifically in forebrain neurons that have a

reduction on 2-AG in these brain areas show a resistance to diet-induced obesity[87]. These transgenic mice also present leanness, elevated energy cost of activity, and hypersensitivity to thermogenesis, which is corrected by reinstating 2-AG activity at CB1R. These results suggest that 2-AG signaling through CB1R regulates the activity of forebrain neural circuits involved in the control of energy metabolism[87].

The intracellular pathways involved in the possible different regulation of food intake by endocannabinoids have not been yet clarified. In this regard, reduced hypothalamic mTOR signaling contributes to the development of hyperphagia, weight gain, and leptin resistance during diet-induced obesity[88]. This signaling pathway is involved in several responses mediated by the endocannabinoid system[20, 63], and further estudies will be required to determine its possible role in the effects of endocannabinoids on food intake and metabolism.

d. Memory modulation

Acute and chronic exposure to cannabis produces dose-related cognitive impairments, most consistently in attention, working memory, verbal learning, and memory functions in animals[31, 89, 90] and humans[91]. These cognitive effects of cannabinoids are due to the expression of cannabinoid receptors in brain areas involved in cognition and the role of the endocannabinoid system in synaptic plasticity[8]. Thus, the hippocampal long-term potentiation was impaired by repeated THC administration, and this effect persisted for 3 days after the last THC injection[92], an observation that fits with the doses and the time-course for the recovery of memory impairment after chronic THC administration[63]. The endocannabinoid system participates in several forms of short- and long-term synaptic plasticity in the hippocampus. Endocannabinoid-mediated short-term plasticity consists of depolarization-induced suppression of inhibition or excitation (DSI or DSE, respectively)[93]. Interestingly, mitochondrial CB1R were

involved in the endocannabinoid-dependent DSI in the hippocampus[18, 94]. The endocannabinoid-mediated long-term inhibition of release (endocannabinoid-LTD) occurs at inhibitory terminals, and facilitates the induction of long-term potentiation (LTP) at excitatory inputs[95].

The cognitive effects of exogenous and endogenous cannabinoids seem to be the result of an unbalance between excitatory and inhibitory inputs that could be explained by the enhanced level of CB1R expression at GABAergic terminals compared to glutamatergic terminals[14]. Indeed, the CB1R in forebrain GABAergic neurons, but not in glutamatergic terminals, were critical for the amnesic-like effects of THC administration[20, 63]. This unbalance between the excitatory and the inhibitory drive could mediate the activation of the mTOR pathway and the protein synthesis machinery in the hippocampus through a glutamatergic mechanism, underlying the long-term memory impairment induced by THC or increased anandamide levels[20, 31, 63, 90]. Indeed, mTOR inhibition blocked the object-recognition memory deficit when co-administered with exogenous or endogenous cannabinoids[20, 31, 63, 90]. An alternative or complementary explanation for the amnesic-like effects could derive from the presence of CB1R in astroglia since mice lacking astrocytic CB1R did not show the working memory impairment produced by exogenous cannabinoids[18]. CB1R would promote the release of glutamate from astroglia, which could then act on perisynaptic NMDARs producing long-term plastic changes[17, 18].

Repeated exposure to cannabinoids is associated to the development of tolerance to most of their central pharmacological effects, due to CB1R down-regulation/desensitization and reduced mRNA expression[96]. The down-regulation of CB1R expression appears with distinct degrees of strength in different brain areas[97] pointing to specific mechanisms of CB1R regulation probably associated to different cellular environments. In this regard, a recent study using conditional knockout mice has revealed that chronic THC treatment produced a stronger down-regulation in

GABAergic CB1R than in principal glutamatergic neurons of the hippocampus[63]. Despite the strong down-regulation, the memory deficits associated to repeated THC administration were maintained, while the same animals presented tolerance to the antinociceptive and hypothermic effects of THC[63].

Anandamide and 2-AG, could play a differential role in synaptic remodeling regulation[98] and cognitive responses[31]. Both, anandamide and 2-AG, mediate long-term forms of synaptic plasticity, such as LTD or LTP[99]. Interestingly, URB597 induces alterations in hippocampal ensemble activity in wake, behaving animals[100] that correlate with the effects of the administration of URB597 in memory consolidation impairment[31]. Anandamide has also been related to memory acquisition phases[101], consolidation and extinction[9]. On the other hand, 2-AG, acting as a retrograde messenger, modulates short- and long-term forms of plasticity in the hippocampus[12]. However, the role of 2-AG in memory formation is quite controversial since both improved levels[102] and impaired cognitive performance[103] have been reported after increasing 2-AG levels. Recent evidences suggest that anandamide and 2-AG could also modulate memory consolidation in a different manner. Thus, a memory deficit was observed in the object recognition and context conditioning tests when the FAAH inhibitor URB597 enhanced the endogenous anandamide levels. In contrast, 2-AG enhanced levels with the MAGL inhibitor JZL184 did not impair memory in these models[31]. However, higher doses of JZL184 impaired memory in the water maze test[104], suggesting that when both FAAH and MAGL enzymes are inhibited by JZL195, a memory deficit similar to that of THC is revealed[105]. All these data indicate the relevance and a possible dichotomy of the two main endocannabinoids in the modulation of memory that could have important therapeutic interest.

3. Conclusions and future directions

A better understanding on the role of the endocannabinoid system in different physiological functions has been possible during the last years due to the generation of new selective pharmacological tools and specific conditional transgenic mice. As summarized in the present review, the endocannabinoid system plays a crucial role in nociception, anxiety, food intake and memory, opening new strategies for therapeutic interventions. Other central responses mediated by the endocannabinoid system that have not been addressed in this review are also of potential therapeutic interest, such as neuroprotection, motor coordination or addiction. The various roles assigned to the endocannabinoid system and the characterization of the molecular complexity of these responses reinforce the idea that this system contains specific targets of therapeutic interest for several neurological disorders.

Multiple pharmacological approaches have been developed to modify the activity of these particular endocannabinoid targets. Early approaches have generated compounds with selective (synthetic) or non-selective (natural or synthetic) agonist activity on CB1R and/or CB2R, as well as selective antagonists of these receptors. More recent approaches have provided specific compounds that enhance the activity of the endocannabinoids by inhibiting the enzymatic degradation or the cell re-uptake.

Finally, uncovering the specific intracellular pathways involved in the responses produced by the activation of the endocannabinoid system has revealed novel approaches to promote combined therapies for improving the beneficial effects obtained from the modulation of the endocannabinoid system minimizing the possible incidence of particular side effects. The elucidation of the neurobiological and cellular mechanisms involved in the different responses mediated by the endocannabinoid system will facilitate the development of novel strategies to target specific components of this system or their intracellular targets for therapeutic purposes.

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Conflict of interest

The authors declare no conflict of interests.

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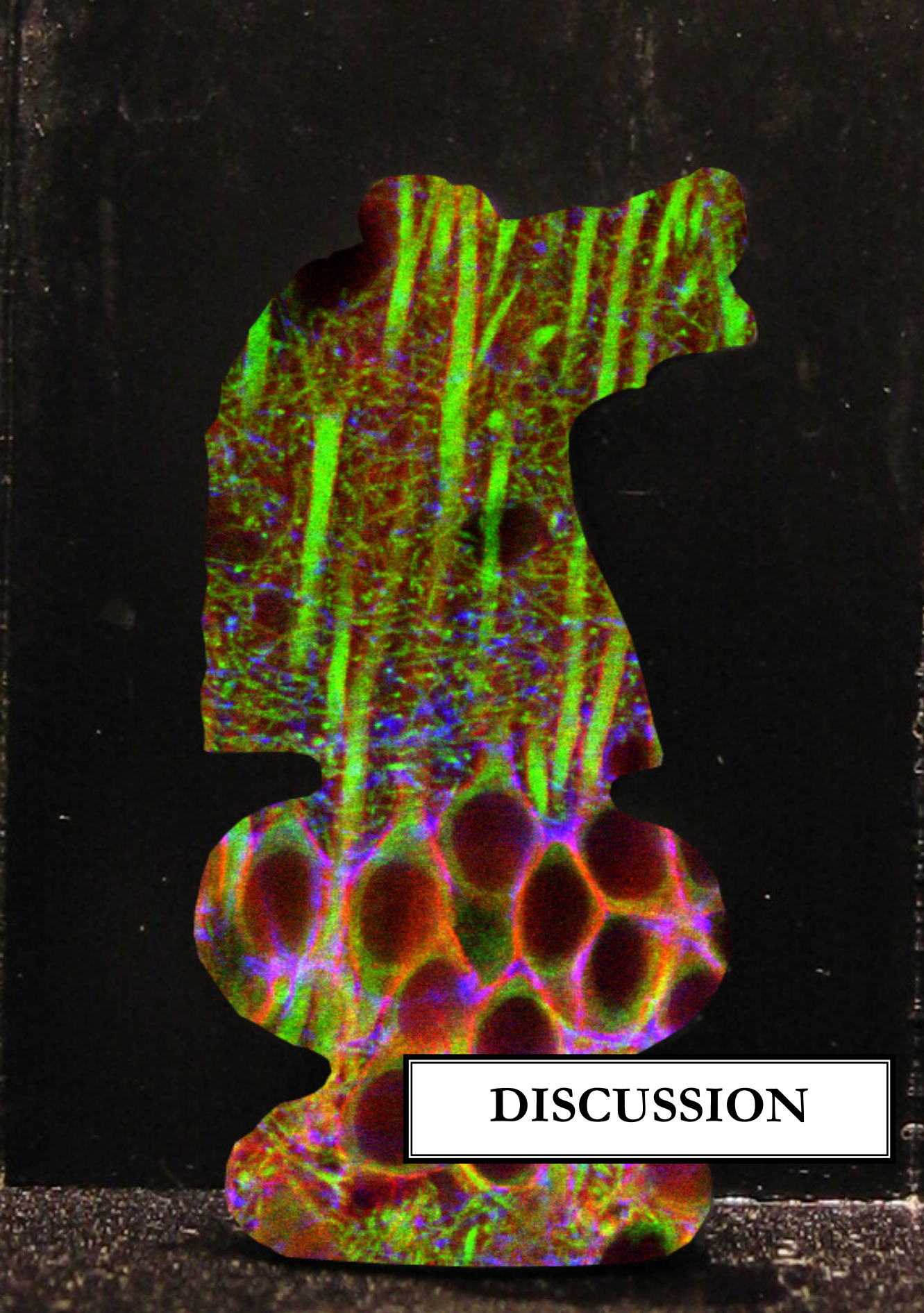


DISCUSSION

1. V-maze: a new protocol to study object recognition memory

The object recognition memory paradigm was first described when scientists reported that rodents spend more time exploring a novel object than a previously explored familiar object (Berlyne et al., 1966). Several advantages exist when comparing this object recognition memory procedure to other tests for cognitive function commonly used. The object recognition memory test takes advantage of the animal's tendency to approach and explore novelty. Therefore, this test does not require long preliminary training and can be achieved after a single training session. This allows a temporal definition of memory formation similar to that obtained with other paradigm, such as the fear conditioning. On the other hand, it does not require exposure to aversive or stressful stimuli stronger than novelty itself, and does not require food or water restriction. Altogether, these factors have contributed to the growing popularity of this procedure (Dere et al., 2007).

The object recognition memory task has been replicated using a variety of apparatus designs and objects in rats and mice. This test has been classically performed in a large open field where animals navigate to explore the objects (Bevins and Besheer, 2006). In our experience, mice performance in this type of setting requires longer habituation periods (several days), several training sessions (Dere et al., 2005), and long-term memory might be difficult to observe (Sik et al., 2003). Moreover, the open field setting results in a large variability at the time of assessing the exploratory activity of the animal (the interaction of

the animal with the object), probably due to spatial and contextual confounds together with the pro-anxiogenic effects of the context. All these factors participate to increase the variability within experimental groups and the need for large groups of rodents (Viola et al., 2010), increasing the overall length of the assay and decreasing its accuracy.

Interestingly, other researchers have introduced the use of a Y-shaped maze to perform the novel object recognition test in rats (Forwood et al., 2005, Winters et al., 2004), but the possible application of this conformation has not been explored in mice to the best of our knowledge. We started to use a V-shaped maze (V-maze) to minimize the context surrounding the objects and to reduce other possible cues different to the objects themselves in a uniform environment, which facilitates the interaction of the animal with the object. Moreover, the arms in the V-maze are relatively short and narrow *(Fig. 26)* reducing the possible anxiety-related bias of the open field situation. Therefore, the V-maze setting maximizes the exploration time of the objects relative to the surrounding context and increases the accuracy of the test. This new behavioral setting has been registered (U200802592) and is under commercial exploitation by Panlab S.A.

This improved setting has been used successfully to evaluate short-term memory and long-term memory depending on the retention time between the training session and the test session (up to 10 min-3 h and 24-48 h, respectively) in inbreed (C57BL/6) and outbreed (CD-1) mice. Moreover, it can be adapted to study different object recognition memory stages, such as acquisition, consolidation, reconsolidation and retrieval *(Figure 26)*. This setting also improves both the sensitivity of the assay for mice as well as the reproducibility, reducing the

habituation and the length of the sessions, and facilitating the cognitive processing.

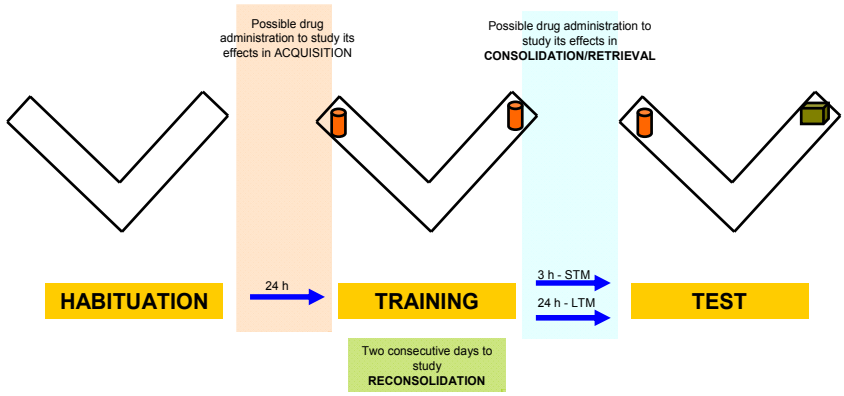


Figure 26. **Scheme of the object recognition protocol**. The protocol consists of three different phases, habituation, training and test, and each phase lasts 9 minutes. Short-term memory or long-term memory can be assessed depending on the retention time between the training and the test session. Different memory phases can be studied: acquisition, consolidation, retrieval and reconsolidation. During the test, the discrimination index is calculated *(DI = (Time in novel object – Time in familiar object) / (Total exploration Time)*. DI values around 0.4 are considered as an indication of good memory. In contrast, DI values under 0.2 are considered as an indication of memory disturbance.

We also set up a modified version of the acute object recognition protocol that allows a repeated assessment of object recognition memory during a chronic treatment. Instead of two identical objects, two distinct objects are placed in the starting training trial. During each test session, a novel object replaces one familiar object, and each testing day served as the training trial for the following day *(Figure 27)*.

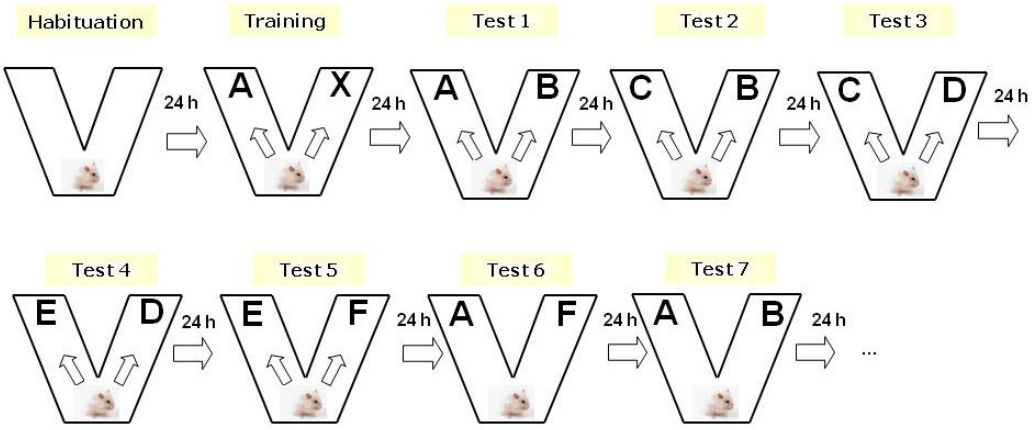


Figure 27. **Chronic object recognition task**. Protocol for the repeat assessment of the effects of drug chronic treatments on long-term memory.

In conclusion, we have validated this new setting and protocol to study the cognitive performance in response to both acute and chronic pharmacological treatments. This protocol has been used in our studies to assess memory in mice after acute and chronic THC administration (Puighermanal et al., 2009, Puighermanal et al., 2013), after the specific enhancement of the two main endocannabinoids (Busquets-Garcia et al., 2011), and in a model of fragile X syndrome (Busquets-Garcia et al., 2013).

2. Mechanisms underlying the memory impairment produced by exogenous and endogenous cannabinoids.

2.1. Molecular basis of acute amnesic-like effects produced by CB1R agonists.

The understanding of the mechanisms by which cannabinoid agonists induce memory impairment can be helpful to elucidate the role of the endocannabinoid system in cognitive function. Our work suggests that the deregulation of the excitatory/inhibitory neurotransmission balance in the hippocampus could be the putative mechanism underlying the deleterious effects of exogenous cannabinoids on memory consolidation. This unbalance results in the activation of signaling pathways that alter the normal protein synthesis in the postsynaptic neuron leading to the memory impairment induced by THC administration (Puighermanal et al., 2009).

CB1R are much more densely expressed on GABA-ergic than glutamatergic axon terminals in the hippocampus (Kawamura et al., 2006, Bellocchio et al., 2010). Moreover, THC has been suggested to act as a full agonist at hippocampal CB1R located on GABA-ergic terminals, while it acts as a partial agonist at glutamatergic CB1R (Laaris et al., 2010). Therefore, THC would preferentially decrease GABA release rather than glutamate release producing a disruption of hippocampal network activity, which is mediated by synchronized GABA-ergic discharges (Hajos et al., 2000, Robbe et al., 2006). In agreement, mutant mice overexpressing the GABA transporter type 1 in neurons, which removes GABA from the synaptic cleft, displayed

memory impairment (Hu et al., 2004, Ma et al., 2001). These results indicate that decreased GABA-ergic tone, as a consequence of increased clearance of GABA from the synaptic cleft, alters memory in the object recognition task. Alternatively, electrophysiological studies show that repetitive low-frequency synaptic stimulation promotes persistent up-regulation of endocannabinoid signaling at CA1 GABA-ergic synapses. Thus, LTD is induced at inhibitory synapses while LTP is facilitated at glutamatergic synapses (Zhu and Lovinger, 2007). All these results demonstrate that the possible unbalance between excitatory and inhibitory transmission produced by cannabinoids could disrupt the normal hippocampal LTP causing the memory impairment.

Our group has also demonstrated that this excitatory/inhibitory unbalance can trigger the activation of the mTOR pathway and the protein synthesis machinery in the hippocampus, underlying the characteristic long-term memory impairment induced by THC. In this regard, we demonstrated that acute systemic THC administration promotes the phosphorylation of different components of the mTOR pathway and of the translational apparatus, such as the ribosomal protein S6 and the eukaryotic initiation factors eIF4E, eIF4G, and eIF4B (Puighermanal et al., 2009). Interestingly, non-amnesic doses of the protein translation inhibitor anisomycin or a specific mTOR inhibitor rapamycin prevent the disruptive effects that THC produces in the memory tasks. These results indicate that mTOR and protein synthesis are required for the long-term amnesic-like effects of THC (Puighermanal et al., 2009). Importantly, it has been demonstrated that the intact function of mTOR and the precise control of translation are

required for the proper memory storage. Thus, both an enhanced or reduced level of activity of the mTOR signaling cascade has been recently correlated to memory disruption (Troca-Marin et al., 2012).

The use of CB1R conditional knockout mice that lack CB1R either in glutamatergic or GABA-ergic neurons (Monory et al., 2006) has revealed important insights into the role of CB1R on memory regulation. Interestingly, the impairing effects of exogenous cannabinoids on long-term memory have been associated to CB1R in GABA-ergic terminals (Puighermanal et al., 2009). Alternative or complementary explanations could come from the recent observation of CB1R in astroglia or mitochondria. Astrocytic CB1R promote the release of glutamate that could then act on perisynaptic NMDARs turning on long-term plastic changes (Navarrete and Araque, 2010). Interestingly, the impairing effects of cannabinoid agonists in working memory and the *in vivo* hippocampal LTD is fully abolished in mice lacking astrocytic CB1R (Han et al., 2012). Recently, intracellular CB1R localized in mitochondria have been also involved in the modulation of endocannabinoid-mediated depolarization-induced suppression of inhibition and LTD in the hippocampus (Benard et al., 2012) suggesting a possible role of these intracellular receptors on memory formation that has to be further studied.

2.2. Differential modulation of memory consolidation by the increase of the two main endocannabinoids.

After studying the memory deficits induced by exogenous cannabinoid agonists, we have investigated the cognitive effects produced by the

selective increase of each one of the two main endocannabinoids. Similarly to THC administration, an increase in the activity of the hippocampal mTOR pathway that correlates with memory impairment was revealed when endogenous anandamide levels are enhanced by the FAAH inhibitor URB597. In line with our results with THC, the NMDA antagonist MK-801 blocked the cognitive impairment produced by URB597, pointing to a similar mechanism. In addition, systemic inhibition of mTOR prevents both the enhanced mTOR signaling and the cognitive deficit promoted by URB597. In contrast, 2-AG enhanced levels with the MAGL inhibitor JZL184 do not induce mTOR activation in the hippocampus nor memory impairment in the novel object recognition and the context recognition memory tasks. These results can be explained by the different efficacy of these two endocannabinoids to stimulate CB1R as well as the duration of the activation of distinct intracellular signaling events in the neuron. Thus, if both endocannabinoids would bind to CB1R located in the same cell type, it is tempting to speculate that anandamide, but not 2-AG, would promote mTOR signaling activation, probably due to this different efficacy to modulate intracellular responses. In this regard, THC would activate CB1R in a similar manner as anandamide does. However, the differences in the activation of CB1R by distinct agonists have to be clarified in the future with more specific approaches.

Based on the immunohistochemical localization of the enzymes involved in the synthesis and degradation of 2-AG (Dinh et al., 2002, Katona et al., 2006), we can speculate that the machinery involved in 2-AG processing is mainly associated with the functional activity of

glutamatergic CB1R in the hippocampus, which are not involved in the memory impairment produced by cannabinoids (Puighermanal et al., 2009). Accordingly, DAGL, the major synthetic enzyme for 2-AG, is mainly expressed in postsynaptic spines directly across from excitatory afferent terminals and it is rarely located near CB1R-expressing perisomatic inhibitory terminals (Katona et al., 2006, Yoshida et al., 2006). Moreover, MAGL is enriched in hippocampal excitatory axon terminals according to the ultrastructural analyses performed in rodents (Ludanyi et al., 2011). On the other hand, anandamide could be mainly involved in the homeostasis of hippocampal GABA-ergic terminals (Kim and Alger, 2010), where CB1R are heavily expressed (Katona et al., 1999). Indeed, NAPE-PLD, the main enzyme synthesizing anandamide, is not found in glutamatergic pyramidal cell bodies and it is mainly located in axons (Egertova et al., 2008, Nyilas et al., 2008, Cristino et al., 2008). Moreover, the FAAH enzyme is placed selectively in the somatodendritic compartment of principal neurons, mainly on intracellular calcium stores (Gulyas et al., 2004). This differential distribution could play an important role in the amnesic-like properties of endocannabinoids.

Our results provide robust evidence for a functional dissociation of the role of each endocannabinoid in memory consolidation. However, other memory stages, such as acquisition or retrieval, might be differentially modulated by the endocannabinoid system because they are sustained by different neurobiological substrates (Romero-Granados et al., 2010). Anandamide has been related to acquisition phases (Murillo-Rodriguez et al., 1998) and memory extinction

(Luchicchi and Pistis, 2012). Interestingly, one study shows that URB597 exerts different effects in learning depending on the memory phase analyzed. Thus, URB597 enhanced memory acquisition, but did not affect memory consolidation (Mazzola et al., 2009). However, this enhancing effect of URB597 on memory acquisition is mediated mainly by another bioactive lipid, oleoylethanolamide, through alpha-type peroxisome proliferator-activated nuclear receptors activation and not by cannabinoid receptors (Mazzola et al., 2009). The role of 2-AG in memory also has been investigated. 2-AG plays a prominent role as a retrograde messenger in the hippocampus, but the enhancement of 2-AG endogenous levels did not affect memory consolidation. However, the role of 2-AG in memory formation is quite controversial because some studies show an improved performance when increasing 2-AG levels (Pan et al., 2011), whereas other studies report a cognitive impairment (Vigano et al., 2009). This discrepancy could be explained by the different models of memory, the different doses and the specificity of the MAGL inhibitor used.

In addition, anandamide and 2-AG exert different specific roles in synaptic remodeling regulation (Katona and Freund, 2012). Both, anandamide and 2-AG mediate long-term forms of synaptic plasticity, such as LTD and for LTP (Robbe et al., 2002). Additionally, an *in vivo* study demonstrates that URB597 induces alterations in hippocampal ensemble activity in awake, behaving animals (Goonawardena et al., 2011). Interestingly, similar results are found by THC or WIN55,212-2 administration in the same study. These results correlate with the amnesic-like effects reveal in our experimental conditions by the administration of URB597.

In contrast with our results, some studies show possible deleterious effects in memory by the specific increase of 2-AG. Thus, high dose of JZL184 impairs short-term memory performance (Wise et al., 2012). These findings are consistent with other results showing that full MAGL inhibition produces a more extensive subset of THC-like effects than that produced by full FAAH inhibition (Long et al., 2009a). However, other studies show that the performance in the object recognition task and acquisition of reference memory in the Morris water maze is enhanced in the knockout mice deficient in MAGL (Pan et al., 2011). In this regard, MAGL inhibition potentiates depolarization-induced suppression of inhibition in neurons of the hippocampus, cerebellum and cingulate cortex (Schlosburg et al., 2010, Pan et al., 2009), brain areas involved in learning and memory processing. These divergent findings indicate that the effects of elevating 2-AG on memory function could depend on different factors, including the task, the dose and the brain region involved. Overall, these findings indicate that while endocannabinoids activate the same receptors, they can produce divergent effects on memory processing that could have important therapeutic relevance. Indeed, when both FAAH and MAGL enzymes are inhibited by JZL195, a memory deficit similar to that of THC is induced in the water maze performance (Wise et al., 2012) demonstrating the crucial role of endocannabinoids in memory formation.

3. Modulation of anxiety-like responses induced by exogenous and endogenous cannabinoids.

Another important aim of this thesis was to study the involvement of the endocannabinoid system in the regulation of anxiety-like responses. Most of the studies have reported that exogenous cannabinoid agonists display biphasic effects, eliciting anxiolytic-like responses at low doses, whereas higher doses induce anxiogenic-like effects. Thus, low doses of the CB1R agonists, nabilone (Onaivi et al., 1999), CP-55,940 (Rey et al., 2012, Marco et al., 2004) and THC (Ashton and Moore, Valjent et al., 2002, Puighermanal et al., 2013) induce an anxiolytic-like effect, whereas higher doses of HU-210, CP-55,940 (Marco et al., 2004, Rey et al., 2012) and THC (Valjent et al., 2002, Puighermanal et al., 2013) produce an anxiogenic-like response in several anxiety paradigms.

One possible mechanism to explain these biphasic effects of cannabinoids could be due to the different activation of CB1R located in distinct neuronal types. Thus, the CB1R located in cortical glutamatergic terminals are crucial for the anxiolytic-like effects of low doses of CP-55,940, whereas the CB1R on the GABA-ergic terminals are required to induce the anxiogenic-like responses (Rey et al., 2012). Low doses of cannabinoids will first affect the CB1R on glutamatergic neurons reducing glutamatergic transmission, leading to an anxiolytic-like effect. On the other hand, a positive allosteric modulator of GABA, GS-39783, counteracts the anxiogenic effects of high doses of CP-55,940 (Rey et al., 2012). This result suggests that a reduction of GABA release mediated by high doses of CP-55,940 could indirectly increase the glutamatergic tone leading to the anxiogenic-like

responses. On the other hand, the unbalance between excitatory/inhibitory inputs could lead to different activation of postsynaptic signaling pathways in brain regions involved in the anxiety modulation, such as the amygdala. Indeed, a high dose of THC, which exerts an anxiogenic-like response, enhances the phosphorylation of p70S6K (T389) in the amygdala, whereas the low dose of THC do not affect the activity of this component of the mTOR signaling (Puighermanal et al., 2013). Notably, only the anxiogenic-like responses triggered by THC were modulated by mTOR activity inhibition demonstrating the different molecular mechanisms involved in this biphasic anxiety-like responses of cannabinoids (Viveros et al., 2005, Ruehle et al., 2012).

On the other hand, we have investigated the effects of the specific increase of the two main endocannabinoids in the regulation of anxiety-like responses. In agreement with previous studies, the specific inhibition of FAAH produces anxiolytic-like effects in several anxiety tests in animals (Gaetani et al., 2009). Accordingly, experiments using FAAHKO mice confirmed that anandamide induces anxiolytic-like effects through the activation of CB1R (Mechoulam and Parker, 2013, Busquets-Garcia et al., 2011). However, the inhibition of FAAH may lead to indirect activation of other targets by the increase of anandamide itself (Di Marzo et al., 2002, Oz, 2006) or other fatty acid amides (N-palmitoylethanolamine and N-oleoylethanolamine)(Moise et al., 2008) that could be involved in these anxiety-like effects. In this regard, TRPV1 has been recently involved in the modulation of anxiety-like behavior by anandamide (Kasckow et al., 2004, Marsch et al., 2007, Rubino et al., 2008). In addition to this, it is important to

mention that anandamide is released in response to anxiogenic situations in the amygdala (Gaetani et al., 2003, Marsicano et al., 2002). Moreover, stress also increases serum concentrations of anandamide in humans and baseline anxiety ratings are negatively correlated with baseline serum concentrations of anandamide (Dlugos et al., 2012). All these data suggest that the enhancement of anandamide levels would represent a physiological mechanism to counteract the behavioral manifestations of anxiety under these stress situations.

We observed that the enhancement of 2-AG levels produces similar effects to anandamide reducing the anxiety-like behavior, according to previous results (Sciolino et al., 2011, Mechoulam and Parker, 2013). However, there are controversial findings regarding the receptor involved in these anxiolytic-like responses. Our study demonstrates that the anxiolytic-like effects of JZL184 are absent in CB2KO mice and are prevented by pre-treatment with the selective CB2R antagonists SR144528 or AM630. Moreover, the administration of JWH133, a selective CB2R agonist, mimics the anxiolytic-like effects of JZL184 revealing a crucial role of CB2R activation on the modulation of anxiety as it has been previously reported using transgenic mice overexpressing CB2R (Garcia-Gutierrez and Manzanares, 2011). In the same study, it is revealed that CB2R might contribute to the regulation of the GABA-ergic system (Garcia-Gutierrez and Manzanares, 2011). In agreement, JWH133 suppresses GABA-ergic inhibitory signaling in the hippocampus, which is blocked by prior administration of AM630 (Morgan et al., 2009). More research must be done to clarify the mechanisms involved since CB2R

located in neurons could participate, but the involvement of microglial or astrocytic cannot be discarded.

In contrast, the anxiolytic-like effects of JZL184 under conditions of high environmental averseness or in the marble burying assay have been prevented by CB1R blockade (Kinsey et al., 2011, Sciolino et al., 2011). The reason of this discrepancy could be the different experimental conditions or the dose of JZL184 used because high doses or chronic treatments could also affect FAAH activity and thereby increase anandamide levels (Long et al., 2009a, Long et al., 2009b, Schlosburg et al., 2010) contributing to a CB1R-mediated suppression of anxiety, as revealed by URB597 administration.

4. Effects of chronic cannabinoid administration

4.1. Behavioral tolerance

Most of the pharmacological effects of cannabinoid agonists undergo tolerance following repeated drug administration. In this regard, we have evaluated the possible development of tolerance to different behavioral responses after chronic cannabinoid treatment. In a first set of experiments, we demonstrate that chronic THC administration induces tolerance to its hypolocomotor, antinociceptive, and hypothermic effects, as previously described (Rubino et al., 2006, Hutcheson et al., 1998). In contrast, no tolerance to the anxiogenic- and amnesic-like effects of repeated THC administration is detected, in agreement with previous behavioral (Boucher et al., 2009) and electrophysiological studies (Hoffman et al., 2007, Fan et al., 2010). Thus, hippocampal LTP is impaired by repeated THC administration

under similar conditions to those used in our study, and this effect persists for 3 days after the last THC injection (Hoffman et al., 2007). This observation fits with the time course for the recovery of object-recognition memory performance reported in our studies. In contrast, other studies have demonstrated the development of tolerance to amnesic-like effects of cannabinoids after a long chronic treatment (Hampson et al., 2003). Therefore, different factors may influence the development of tolerance to cognitive impairment induced by cannabinoids, such as the dose, the duration of the treatment or the task evaluated.

Interestingly, temsirolimus pre-treatment blocks the object-recognition memory deficit when co-administered with chronic THC. The Akt/mTOR pathway has been associated with the modulation of structural plasticity in dendrites and dendritic spines (Swiech et al., 2008) and the activation of this signaling pathway by THC could alter synaptic plasticity mechanisms, impairing the object-recognition memory consolidation (Puighermanal et al., 2009). In agreement, THC has been reported to inhibit activity-dependent synaptic loss *in vitro* (Kim et al., 2008), a relevant process for structural plasticity (Bruel-Jungerman et al., 2007), for which cannabinoids do not develop tolerance (Kim et al., 2008). The observation that temsirolimus do not resolve the residual memory deficit when administered after chronic THC exposure suggests that the resulting alterations after chronic THC administration are no longer sensitive to mTOR modulation. Our previous results and the crucial role of mTOR in the regulation of translation (Hoeffer and Klann, 2010) reinforce the idea that the mTOR-insensitive residual memory deficit could be the result of

mTOR-dependent plastic changes that are temporarily stabilized. Together, these data suggest that an appropriate synaptic plasticity is necessary for the object recognition task, and THC could alter synaptic plasticity by modulating mTOR signaling leading to the amnesic-like effects.

All these data suggest that the medicinal cannabis, such as Cesamet (nabilone), Marinol (THC), and Sativex (THC with cannabidiol) (Pertwee, 2012), combined with an mTOR signaling inhibitor like temsirolimus or rapamycin could represent an interesting therapeutic approach to minimize important side effects, such as the anxiogenic- and/or amnesic-like responses. In this regard, there is previous evidence that the benefit-to-risk ratio of a cannabinoid receptor agonist can be improved by administering together with a second drug (Pertwee, 2012).

On the other hand, we have also investigated the possible development of tolerance by the chronic exposure to FAAH and MAGL inhibitors. The chronic administration of URB597 or JZL184 do not result in a reduction of their antinociceptive and anxiolytic-like effects, highlighting their potential interest as novel therapeutic tools. This lack of tolerance to the antinociceptive effects of chronic URB597 and JZL184 administration is in agreement with the absence of tolerance to non opioid stress-induced analgesia, an effect mediated by the endocannabinoid system, where both endocannabinoids seem to be involved (Hohmann et al., 2005). In contrast, the antinociceptive effects induced by high doses of JZL184 developed tolerance after chronic treatment (Schlosburg et al., 2010). This discrepancy with our findings could be explained by the use of a very high dose of JZL184

(40 mg/kg compared with 8 mg/kg in our study), which results in a much higher increase in the levels of 2-AG than in our study (about 10 times the control levels compared with 5 times in our study) that could affect CB1R functionality. In fact, a recent study from the same authors demonstrates that repeated administration of a low dose JZL184 maintains its antinociceptive actions in the chronic-constriction injury of the sciatic nerve neuropathic pain model and the protective effects in a model of NSAID-induced gastric hemorrhages (Kinsey et al., 2013).

Additionally, we observe that chronic inhibition of FAAH produced a similar amnesic-like effect to that observed after an acute intervention with URB597 or THC. This effect is also mediated through the activation of the mTOR pathway caused by a predominant glutamatergic transmission. Interestingly, JZL184 did not affect hippocampal mTOR signaling and did not significantly impair memory consolidation either after acute or chronic administration. Therefore, our data suggest that a controlled MAGL inhibition could be more relevant from a therapeutic point of view than FAAH inhibition considering the possible avoidance of the cognitive deficits produced by the activation of CB1R.

4.2. Mechanisms underlying tolerance

The mechanisms underlying the behavioral tolerance produced by cannabinoids have not been yet fully clarified. Several studies indicate that chronic cannabinoid treatment causes changes in CB1R that include CB1R down-regulation and functional tolerance in discrete brain regions (Breivogel et al., 1997, Sim-Selley, 2003). This down-

regulation, although generalized in the brain, runs with different degrees of intensity in distinct brain areas (Sim-Selley, 2003) pointing to specific mechanisms of regulation probably associated to different cellular environments. In this thesis, we have investigated this hypothesis by giving chronic THC treatment to different CB1R conditional KO mice and we have also performed chronic treatments with the specific FAAH and MAGL inhibitors.

We have observed that the down-regulation of hippocampal CB1R induced after chronic THC exposure is higher in CB1R located on hippocampal GABA-ergic than in glutamatergic neurons. Accordingly, previous electrophysiological results report the development of tolerance to GABA release inhibition, but not to glutamate release inhibition after similar chronic THC exposure (Hoffman et al., 2007). We previously showed that the amnesic-like effects of THC are mediated by GABA-ergic CB1R (Puighermanal et al., 2009) and we hypothesized that no down-regulation would be expected in this neuronal type. However, the GABA-ergic CB1R population was heavily down-regulated by chronic THC compared to the glutamatergic CB1R population. These unexpected results, brought us to hypothesize that the more pronounced down-regulation in GABA-ergic CB1R than in glutamatergic CB1R might not be sufficient to influence the unbalancing effect of THC on the excitatory/inhibitory presynaptic control due to the higher CB1R levels in GABA-ergic neurons (Kawamura et al., 2006). A possible explanation to this different cell specific down-regulation could come from the observation that the minority of CB1R on glutamatergic neurons is paradoxically several fold more strongly coupled to G protein

signaling than GABA-ergic CB1R (Steindel et al., 2013). This differential coupling together with our observations raises the possibility of designing novel cannabinoid ligands that differentially activate only a subset of physiological effects of CB1R stimulation, thereby optimizing the therapeutic action. Future research must clarify the precise CB1R localization in specific cellular types that could be involved in distinct physiological and pathological functions.

We show that the doses of URB597 and JZL184 used in our chronic treatment did not reduce the expression of CB1R in the hippocampus. In agreement, chronic administration of anandamide produces less cellular adaptations, as well as less tolerance to antinociception, catalepsy, and hypothermia than chronic THC administration in FAAH KO mice (Falenski et al., 2010). However, the effect of JZL184 on CB1R expression and function in brain depends on the dose used. Interestingly, high doses of JZL184 produce a strong down-regulation and desensitization of central CB1R (Schlosburg et al., 2010), whereas the same authors published a study demonstrating that lower doses of this specific inhibitor does not produce CB1R down-regulation (Kinsey et al., 2013). This recent study confirms our hypothesis that CB1R functional tolerance occurs following repeated administration of high doses of JZL184, but not after repeated administration of low doses. Therefore, the administration of these low doses of inhibitors of endocannabinoid-degrading enzymes may be therapeutically interesting based in two major key points. First, these inhibitors can be used to treat anxiety and pain, mainly due to the lack of tolerance and other important side effects, compared to classical cannabinoid agonists. Second, the use of these drugs highlights one of the main properties of the endocannabinoid system

that is the release of endocannabinoids only on demand in specific tissues and brain areas.

5. Targeting the endocannabinoid system as a possible new therapeutic strategy in the fragile X syndrome.

In the last part of this thesis, we take profit of our previous work revealing the mechanisms involved in different functions of the endocannabinoid system to evaluate the possible modulation of several phenotypes found in a specific genetic disease, the fragile X syndrome. In fact, the endocannabinoid system has been recently related to the pathophysiology of this syndrome (Jung et al., 2012, Zhang and Alger, 2010). Interestingly, we have found that CB1R blockade in Fmr1KO mice, an animal model of the fragile X syndrome, through pharmacological and genetic approaches, normalizes the cognitive impairment, nociceptive desensitization, susceptibility to audiogenic seizures, over-activated mTOR signaling, and altered spine morphology, while pharmacological blockade of CB2R normalized anxiolytic-like behavior and seizure susceptibility. Some of these traits were also reversed by pharmacological inhibition of mTOR or mGluR5. Thus, we demonstrate for the first time that cannabinoid receptor blockade is a potential therapeutic approach to normalize specific alterations in fragile X syndrome.

We observed that CB1R blockade reverses the memory impairment revealed in the Fmr1KO mice pointing to the crucial role of CB1R activity in this behavioral manifestation of fragile X syndrome. Moreover, the intra-hippocampal administration of rimonabant

induces the same effect as the systemic administration revealing the important role of hippocampal CB1R in this memory deficit. In addition, the combination of mGluR5 and CB1R antagonists improve the performance in the memory test of Fmr1KO mice compared with both treatments separately pointing to a complementary involvement of both mechanims. These results discard the idea that both receptors lie in the same pathway to modulate memory consolidation in fragile X syndrome.

Recent studies propose that the lost of FMRP expression affects the efficacy of mGluR5-driven endocannabinoid production machinery in different brain areas (Zhang and Alger, 2010). In our study, we did not detect differences between WT and Fmr1KO mice in brain basal levels of the two main endocannabinoids, nor in the hippocampal expression of several components of the endocannabinoid system in agreement with previous studies (Zhang and Alger, 2010). In addition, we have demonstrated that FMRP does not significantly affect the machinery responsible for endocannabinoid modulation of inhibitory transmission and long-term synaptic plasticity in hippocampal CA1 area. Overall, we did not detect any basal difference in the activity of the endocannabinoid system between WT and Fmr1KO mice. However, these results do not exclude a possible alteration of this system in the fragile X syndrome and perhaps more detailed analysis will be necessary in order to find these possible differences. Indeed, a recent specific study has found that the macromolecular complex that links mGluR5 to the DAGL is compromised in the glutamatergic synapses in the ventral striatum and prefrontal cortex of Fmr1KO mice. These changes were associated to impaired eCB-LTD (Jung et

al., 2012). Recently, using autaptic hippocampal neurons cultured from Fmr1KO mice, it has also been demonstrated that the deletion of Fmr1 gene could modulate the endocannabinoid system in the glutamatergic synapses (Straiker et al., 2013). However, whether this or another alteration is present in GABA-ergic synapses has not been studied yet. To explain our results, we favor the hypothesis that CB1R blockade is acting preferentially in the GABA-ergic neurons, although the detailed characterization of the endocannabinoid system in these synapses is still lacking.

We have also demonstrated that the hippocampus of Fmr1KO mice exhibits a marked increase in the phosphorylation of p70S6K (T389) specifically in CA1 pyramidal neurons, but not in GABA-ergic interneurons. Moreover, the phosphorylation status of two kinases of this mTOR pathway in brain homogenates is specifically enhanced in the hippocampus, but not in other brain regions such as the frontal cortex, striatum, amygdala and cerebellum. Interestingly, both CB1R and mGluR5 blockade normalize the overactivation of the mTOR pathway correcting also the memory deficit. The specific activation of the mTOR pathway in the pyramidal neurons of the hippocampus and its relation to the memory impairment is reminiscent of similar results previously observed after acute THC administration (Puighermanal et al., 2009) and enhanced anandamide levels (Busquets-Garcia et al., 2011). This can explain the efficacy of rimonabant-mediated CB1R blockade to reverse the cognitive deficit in Fmr1KO mice. The fact that the mTOR inhibitor temsirolimus also prevented the object-recognition memory impairment in Fmr1KO mice reinforces the concept that mTOR signaling over-activation plays a key role in this

deficiency, as previously proposed (Troca-Marin et al., 2012, Bhattacharya et al., 2012). Overall, these data suggest the interest of using these pharmacological approaches as a potential therapeutic strategy for fragile X syndrome.

Based on the literature and our experimental data, we hypothesize that there is an unbalance between glutamatergic and GABA-ergic transmission in the hippocampus of fragile X syndrome towards an enhanced excitatory input. Given that CA1 hippocampal GABA-ergic presynaptic membranes display 10-20 fold heavier expression of CB1R than glutamatergic ones (Kawamura et al., 2006), rimonabant might help normalizing this unbalance altered in fragile X syndrome, leading to an improvement on several fragile X syndrome traits. The effect of rimonabant on this balance may also fit with other therapeutic approaches aiming to reestablish the excitatory/inhibitory balance, such as the mGluR5 antagonist CTEP (Michalon et al., 2012), or the NMDA receptor antagonist memantine (Wei et al., 2012), both reducing the excitatory drive, or the $GABA_B$ receptor agonist arbaclofen (Henderson et al., 2012, Berry-Kravis et al., 2012), increasing the inhibitory drive.

Another characteristic manifestation on fragile X syndrome is the alteration in the morphology of the dendritic spines. Interestingly, CB1R have been recently involved in the modulation of dendritic spine formation. Thus, a recent study associates the neuroplastic modifications induced by highly palatable isocaloric food with the activity of the CB1R (Guegan et al., 2013) suggesting a crucial role of CB1R in this process. In our study, we demonstrate that the overall enhanced dendritic spine density of CA1 pyramidal neurons in

Fmr1KO mice was also normalized by rimonabant chronic treatment. Previous findings also reverse this distinctive phenotype observed in fragile X syndrome by other treatments, such as chronic mGluR5 treatment (Michalon et al., 2012). Interestingly, when spines are classified based on their morphology, rimonabant-treated Fmr1KO mice show a decrease in thin/stubby (immature) spines and an increase in mushroom/wide (mature) spines compared to vehicle-treated Fmr1KO mice. Deficits in the maturation of the spines are also observed in other pathologies, such as Down syndrome, Rett's syndrome (Fiala et al., 2002) and other autistic-like disorders (Huang et al., 2002b). These findings raised the interest to assess the CB1R blockade as a possible therapeutic tool on these diseases.

Fragile X syndrome patients also present alterations in anxiety behaviors (Eadie et al., 2009), pain sensation and increased susceptibility to suffer seizures (Qiu et al., 2008). Interestingly, CB2R blockade readily normalizes the reduced-anxiety phenotype observed in the mutant mice. In this regard, we previously showed that the anxiolytic-like effects produced by an enhanced levels of 2-AG are also blocked by the CB2R antagonist AM630 (Busquets-Garcia et al., 2011). Therefore, we conclude that the reduced anxiety phenotype in Fmr1KO mice may result from the CB2R activation although the specific activity of this receptor in the mutant mice has to be investigated in the future. However, the exact mechanisms involving CB2R in this action in fragile X syndrome is still unclear. On the other hand, the decreased antinociception, which is relevant to the self-injurious behavior that often appears in fragile X syndrome (Symons et al., 2003), is completely normalized only by rimonabant treatment

or by the genetic reduction of CB1R. In agreement, an enhanced endocannabinoid tone on CB1R has been reported to limit both peripheral and central nociceptive sensitization (Guindon and Hohmann, 2009).

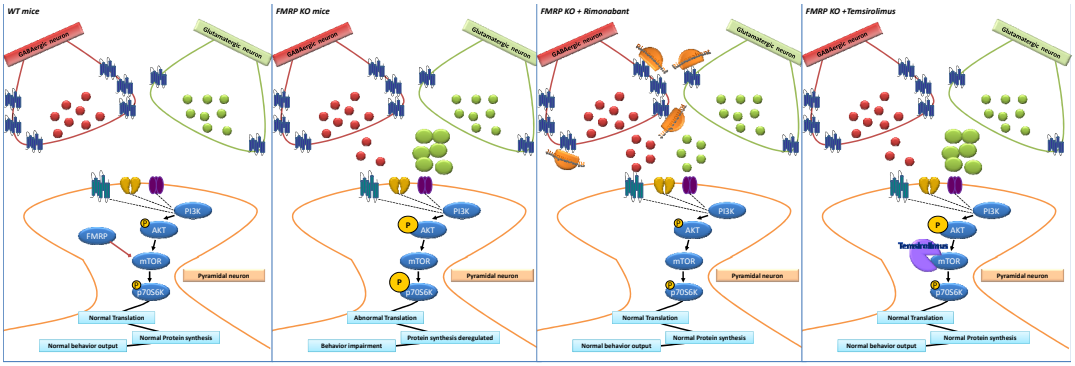


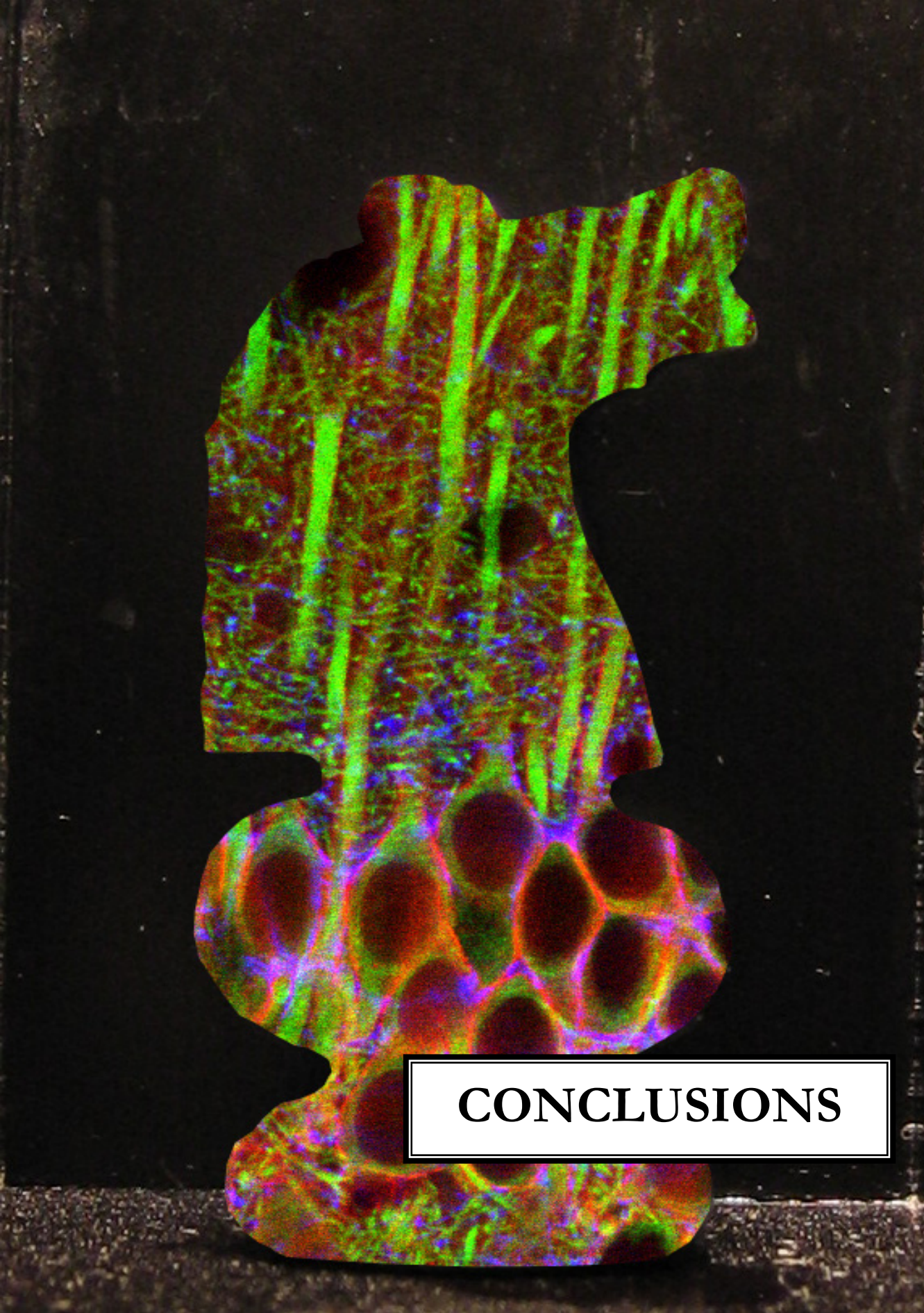
Figure 28. **Schematic diagrams showing the therapeutic use of rimonabant and temsirolimus in the hippocampal fragile X syndrome synapse.** A) In the hippocampus CB1R are mainly localized in GABAergic terminals and to a minor extent in glutamatergic terminals where they modulate neurotransmitter release. FMRP regulates the translation and synthesis of several Akt-mTOR pathway proteins that will contribute to the normal behavioral output. B) In fragile X syndrome, an excitatory/inhibitory unbalance leads to the overactivation of the mTOR pathway producing an aberrant synaptic plasticity that could explain the memory impairment observed in these mice C) Rimonabant and CB1R genetic attenuation contribute to the normalization of the excitatory/inhibitory balance in the hippocampus, leading to the normalization of mTOR signaling, and the behavioral performance in the cognitive test. D) Temsirolimus, by acting directly as an mTOR inhibitor, improves the cognitive performance under conditions where the excitatory/inhibitory unbalance persists *(Busquets-Garcia et al., 2013)*.

Finally, the enhanced sensitivity to audiogenic seizures in Fmr1KO mice (Chen and Toth, 2001) was also decreased by rimonabant, AM630, temsirolimus and by the genetic reduction of CB1R, similarly to the effect previously reported with mGluR5 antagonist (Michalon et

al., 2012). Importantly, these results demonstrate, for the first time, the involvement of CB2R and mTOR signaling in the susceptibility to seizures revealing a new central effect of these targets.

Taken together our results reveal the involvement of the endocannabinoid system in specific behavioral, synaptic and molecular manifestations of fragile X syndrome. In addition, our data point to the endocannabinoid system and mTOR pathway as potential therapeutic targets for treating fragile X syndrome *(Figure 28)*. In contrast, a recent study shows the normalization in Fmr1KO mice of synaptic alterations in ventral striatum and prefrontal cortex and behavioral abnormalities in locomotion and anxiety by the enhancement of 2-AG signaling (Jung et al., 2012). These results may seem contradictory to our data because these authors proposed that activating CB1R could reverse some of the fragile X syndrome alterations whereas we propose in our work the CB1R blockade as a promising therapeutic strategy. However, different aspects must be taken into account to understand these apparent discrepancies. First, the age of the animals (2 months in the previous study and 4-5 months in our study) is crucial because the possible alteration of the endocannabinoid system in these Fmr1KO mice could be progressive. Second, the enhancement of 2-AG levels is not the same as giving a direct agonist, because the agonist will act in all brain regions whereas the endocannabinoid would act in a time- and site-specific manner. Moreover, the dose used of JZL184 in this previous study (Jung et al., 2012) was high enough to act in other possible targets different to the MAGL enzyme (Long et al., 2009a). Finally, the precise subcellular expression of the endocannabinoid system in a physiological or

pathological situation, which is the case of the fragile X syndrome, is also important in order to understand the mechanism that could underlie each phenotype. For example, an alteration of the machinery to produce 2-AG has been proposed in the glutamatergic synapses of mutant mice (Jung et al., 2012) whereas the state of this system in other neuronal populations have not been yet investigated. Importantly, it is clear that the endocannabinoid system plays a crucial role in the pathophysiology of fragile X syndrome and the possible explanations for these apparent discrepancies must be further investigated in the future. This system could also modulate other diseases and its regulation may become a potential therapeutic approach.



CONCLUSIONS

CONCLUSIONS

The main conclusions of the work presented in this thesis are:

1. The novel V-shaped maze allowed reliability for testing in the object recognition paradigm episodic memory and its modulation by pharmacological, genetic or age-related modifications.

2. The enhancement of anandamide levels by URB597 promoted memory deficits through similar mechanisms to those described for THC, while JZL184-enhanced 2-AG levels did not alter memory consolidation.

3. URB597 and JZL184 administration induced anxiolytic-like effects through the activation of different cannabinoid receptors, CB1R and CB2R, respectively.

4. The CB2R agonist JWH133 produced a dose-dependent anxiolytic-like effect pointing to this receptor as a novel target for the treatment of anxiety-related disorders.

5. The effects in memory consolidation, anxiety, and antinociception were maintained after chronic administration of low doses of URB597 and JZL184, altogether pointing to MAGL inhibition as a promising target for therapeutic purposes.

6. A clear tolerance to THC-induced anxiolysis, hypothermia, hypolocomotion, and antinociception was developed after chronic treatment, but not to its anxiogenic- and amnesic-like effects.

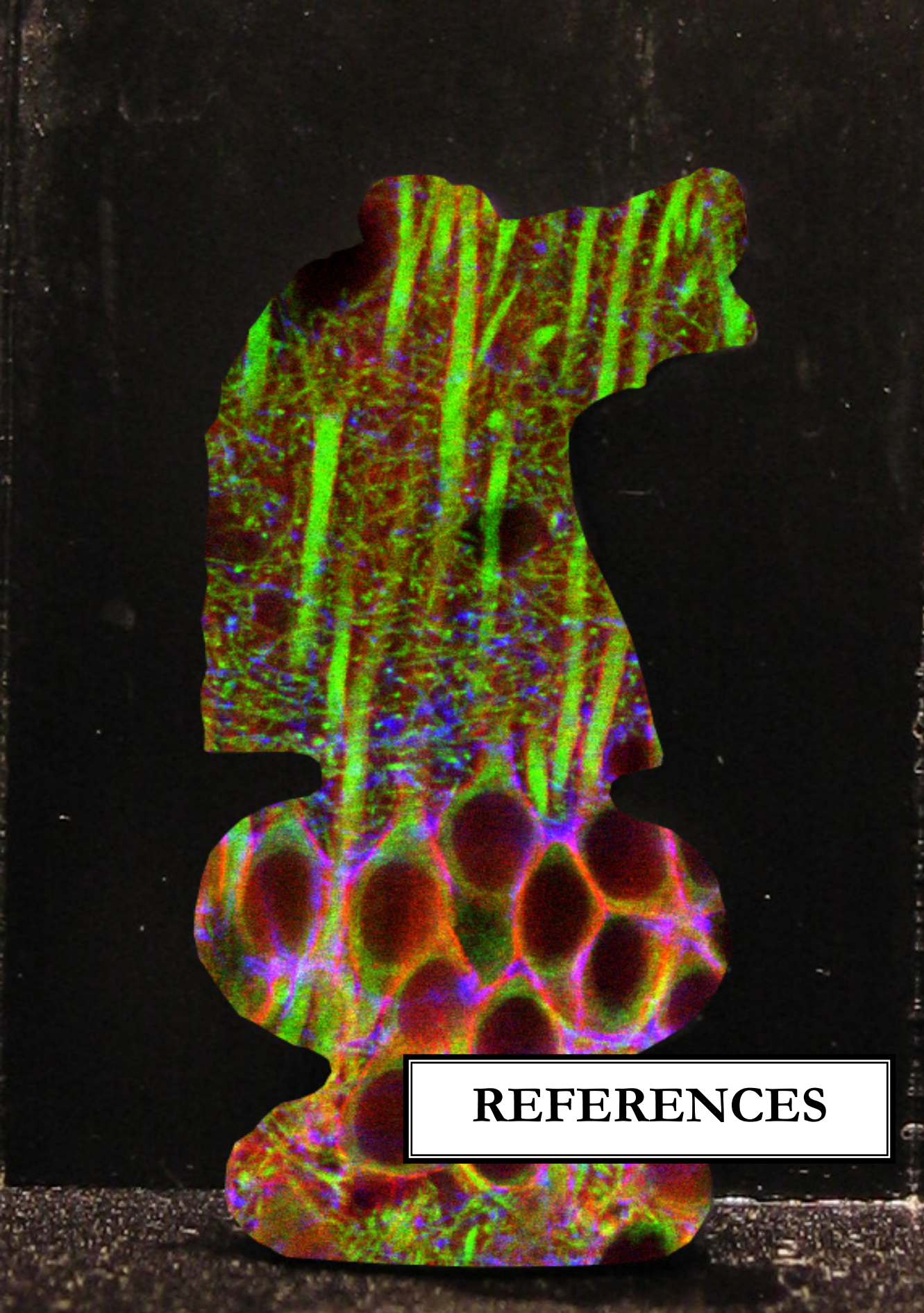
7. GABA-ergic CB1R are mainly downregulated under chronic THC treatment conditions, and GABA–CB1KO mice did not develop cognitive deficits after chronic THC exposure.

8. mTOR inhibition by temsirolimus allows the segregation of the potential beneficial effects of cannabinoid agonists, including the anxiolytic and antinociceptive effects, from the negative side effects, such as anxiogenic- and amnesic-like responses.

9. The endocannabinoid system is a suitable target to normalize specific behavioral, synaptic, cellullar and molecular manifestations of fragile X syndrome, including cognitive impairment, decreased nociceptive response, increased susceptibility to audiogenic seizures, overactivation of the mTOR pathway and the alteration in dendritic spines in the hippocampus.

10. CB2R has an important role in the regulation of anxiolytic-like behavior and increased susceptibility to audiogenic seizures found in fragile X syndrome.

11. The endocannabinoid system and mTOR pathway are potential targets for the development of new therapeutic approaches in fragile X syndrome.



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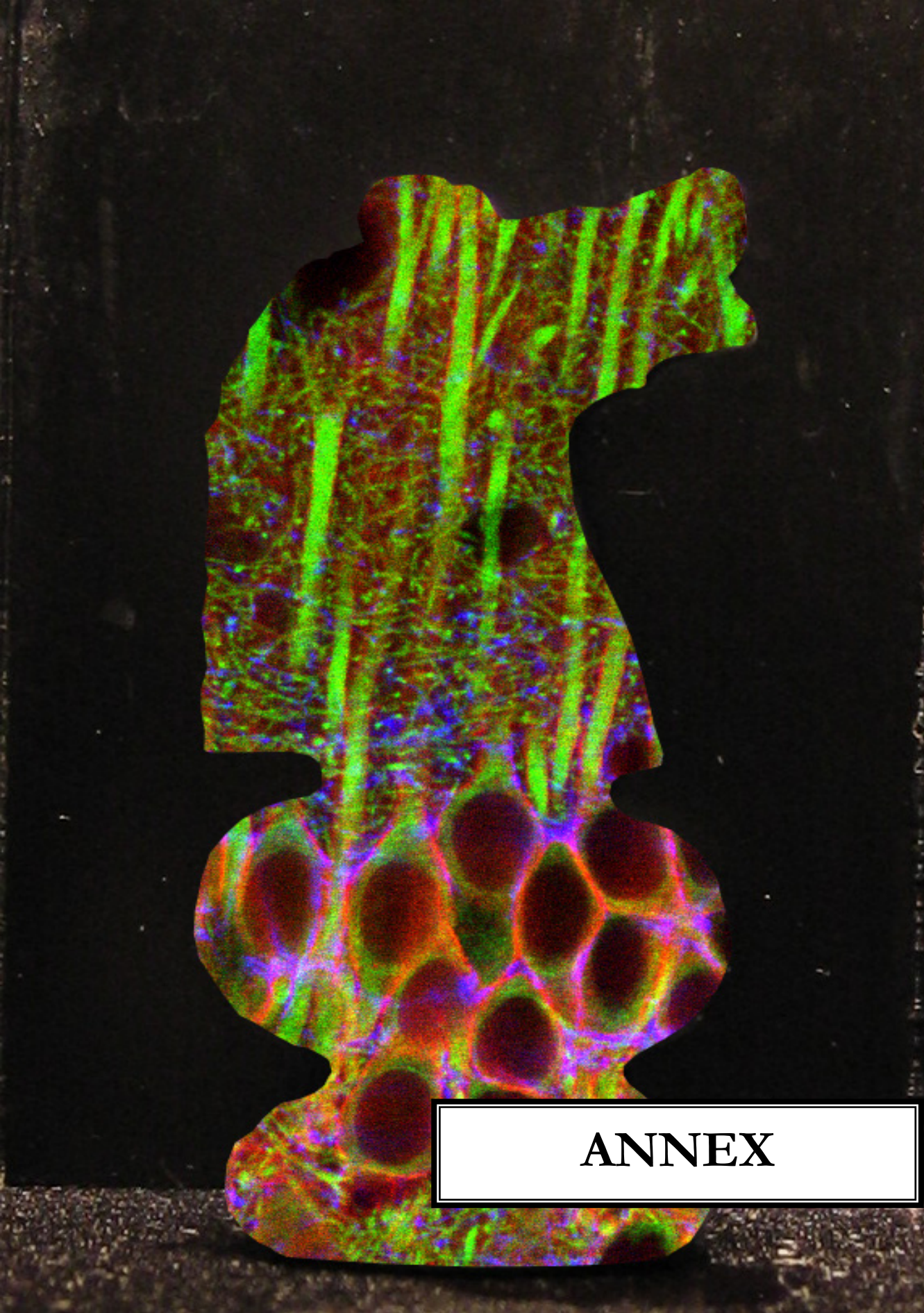

ANNEX

ARTICLE 1

Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling

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ARTICLE 2

Cellular and intracellular mechanisms involved in the cognitive impairment of cannabinoids.

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ARTICLE 3

Mechanisms underlying the cerebellar deficits produced by repeated cannabis exposure

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Mechanisms underlying the cerebellar deficits produced by repeated cannabis exposure

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Conflit of interest

The authors have declared that no conflict of interest exists.

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Abstract

Chronic cannabis exposure has been associated to cerebellar dysfunction in humans but the neurobiological mechanisms involved remain unknown. We observed that sub-chronic delta9-tetrahydrocannabinol (THC, 5 and 20 mg/kg) in mice activates cerebellar microglia and increases the expression of particular neuroinflammatory markers, including IL-1β. This neuroinflammatory phenotype correlated with deficits in cerebellar conditioned learning and fine motor coordination. CB1 cannabinoid receptor (CB1R) downregulation in the cerebellar molecular layer promoted by sub-chronic THC exposure plays a key role in this neuroinflammatory process since a similar phenotype was readily detectable in the cerebellum of CB1R constitutive KO mice (*CB1*$^{-/-}$) and in mice lacking CB1R in the cerebellar parallel fibers (*CB1α6*$^{-}$). An enhanced expression of CB2 cannabinoid receptor and IL-1β mRNA was detected under neuroinflammatory conditions cerebellar cells positive for the microglial activation marker CD11b. The immunosuppressant minocycline and an inhibitor of IL-1β receptor signaling (IL-1 receptor antagonist) prevented the deficits in cerebellar function observed in *CB1*$^{-/-}$ and THC withdrawn mice. Our results suggest that cerebellar microglial activation is crucial for cerebellar-dependent functions, and plays a role in the cerebellar deficits induced by repeated cannabis exposure.

Introduction

Cannabis sativa preparations are the most consumed illicit drugs for recreational purposes(1). Delta9-tetrahydrocannabinol (THC) constitutes the main psychoactive component in those preparations(2). THC acts as a partial agonist of CB1 and CB2 cannabinoid receptors (CB1R and CB2R). CB1R are widely expressed in the brain where they control the release of neurotransmitters at the presynaptic level(3). In contrast, CB2R have been mainly localized in the immune system(4) and show a modest expression in the healthy brain in neurons (5) and microglia(6,7).

Chronic THC exposure induces adaptive changes in the central nervous system (CNS) typified by a down-regulation of CB1R in animal models(8) and humans(9), but the effect of such exposure on CB2R is largely unknown. The marked decrease in the expression, density and functional activity of CB1R after chronic THC differ between brain areas(8). This down-regulation is particularly relevant in the cerebellum, although it has been demonstrated that at least 3 d of daily CB1R agonist administration are required before reductions were detectable(10). In the cerebellum, CB1R are highly expressed in the molecular layer, specifically in the parallel fiber terminals onto Purkinje cells(11) where they regulate long-term synaptic plasticity processes at these glutamatergic synapses(12). Indeed, long-term depression (LTD) at these synapses is thought to be predominant for the cerebellar-dependent conditioned learning, specifically in delayed eyeblink conditioning(13). Thus, CB1R inhibit glutamate release in the cerebellar cortex(14) and the decrease in CB1R activity after sub-chronic cannabinoid exposure results in an enhancement of the glutamatergic postsynaptic activity(14). Accordingly, repeated exposure to cannabis affects cerebellar associative learning in humans as detected in the delayed eyeblink conditioning paradigm(15), while forebrain function measured by the trace eyeblink conditioning paradigm is not affected(16). Similarly, constitutive KO mice lacking CB1R show a complete deficit in the acquisition of

delayed eyeblink conditioning, but not on the trace eyeblink conditioning paradigm(17). Furthermore, when current and former cannabis users were compared in cerebellum-dependent delayed eyeblink conditioning, a disruptive effect in timing-related synaptic plasticity within the cerebellum persisted after the cessation of cannabis consumption(18). Altogether, cerebellar function is largely affected by CB1R de-regulation and cannabis comsumption (19).

Microglial cells are the main immune effector cells of the CNS and express low if any CB1R and CB2R in resting homeostatic conditions, while they express CB2R at detectable levels when microglia is activated (20). These cells are essential for the normal function of the mature CNS since they regulate innate immunity and provide extensive and continuous surveillance of parenchyma and synaptic contacts(21,22,23). In the healthy adult brain, resting microglial cells are characterized by a small soma with fine, highly ramified branches and low expression of surface antigens(24). Following brain stress or injury, local changes in the extracellular milieu promote a change in microglial phenotype, referred to as microglial activation. As microglial cells do not receive direct synaptic inputs, it has been proposed that neurotransmitter receptors expressed by microglia might induce and control this activation(25). For example, microglia expresses a variety of these receptors including those for glutamate and GABA(26) that modulate their functional response(27). In this context, it is known that glutamate, the main excitatory neurotransmitter, acting on glutamate receptors expressed by microglial cells plays a crucial role in classical microglial activation involving IL-1β enhanced expression (26,28). This process includes morphological changes from the resting phenotype to an amoeboid morphology accompanied by changes in the expression of pro-inflammatory and anti-inflammatory genes(29,30).

In this study, we report that sub-chronic THC exposure activates cerebellar microglia. This activation, revealed by changes in the microglial phenotype and by an enhanced CD11b and

IL-1β expression, correlates with the down-regulation of cerebellar CB1R in the molecular layer. The localized neuroinflammatory status described herein has profound relevance in cerebellar-dependent conditioned learning and motor coordination performance. These data suggest that the cerebellar neuronal/glial circuitry is controlled by CB1R in the molecular layer of the cerebellum and the impairment of this mechanism leads to functional alteration of this brain area. Considering that the neuronal circuits involved in this response are similar in mice and human, we hypothesize that the mechanisms described herein would be directly involved in the cerebellar deficits recently reported in chronic cannabis users.

Results

Sub-chronic THC results in conditioned cerebellar learning deficit and motor coordination alteration

We followed a short schedule of systemic THC administration known to produce cannabinoid physical dependence(31) and examined whether cerebellar function would be compromised after THC exposure. Mice received THC (1, 2.5, 5, 20 mg/kg, i.p.) twice daily during 5 d, and once on the sixth day. We used the delayed eyeblink conditioning paradigm, since the cerebellum contributes critically to the acquisition(32) and performance(33) of this task. This learning model involves the same neuronal circuits in rodents and humans(34) and is specifically affected in current and former cannabis smokers(15,18). The first conditioning session was performed on the last day of the THC (or vehicle) treatment starting 4 h after the last drug administration (Supplemetal Figure 1A). The percentage of conditioned responses was measured every day for 9 d. Mice receiving sub-chronic THC at 1, 2.5, 5 or 20 mg/kg showed a dose-dependent deficit in cerebellar associative learning (Figure 1A). Specifically, this deficit was sustained from the third to the eighth day after treatment cessation in the 5 mg/kg (THC-5) or 20 mg/kg (THC-20) groups, while was only revealed on the fifth day in the THC-2.5 group (Figure 1A). In contrast, the conditioned learning performance in the THC-1 group was similar to the vehicle group (Figure 1A). Considering these results, we studied the motor coordination skills on the fifth day after THC cessation in a different set of mice using the accelerating rotarod (Supplemental Figure 2) and the coat-hanger test (Figure 1B). Accelerating rotarod analysis showed a learning impairment in this coordination task in THC-withdrawn mice in comparison with the control group at the same doses revealed in the conditioning paradigm (5 and 20 mg/kg) (Supplemental Figure 2). More robust results were observed using the coat-hanger test (Figure 1B), a more demanding test which allowed for the detection of fine alterations in the motor coordination function. Five days after cessation of

sub-chronic THC treatment, mice showed a dose-dependent alteration in several coordination parameters (fall latency, number of movements along the hanger and extreme latency) in comparison with vehicle treated mice. Significant effects were revealed at the same doses of THC (5 and 20 mg/kg) as in the previous paradigms (Figure 1B). Instead, no significant differences between groups were observed when equilibrium was assessed with the rod test (Supplemental Figure 3). Together, these data revealed the cerebellar deficit associated to THC exposure and subsequent withdrawal.

THC withdrawal produces molecular and cellular signs of cerebellar neuroinflammation

Five days after THC treatment cessation, cerebellar homogenates were analyzed by western blot and showed a THC dose-dependent increase in the expression of the microglial activation marker CD11b and a decreased expression of CB1R (Figure 2A). As revealed by immunofluorescence staining and CB1R intensity analysis, the decrease in CB1R expression occurred mainly in the cerebellar molecular layer of THC-withdrawn mice (5 and 20 mg/kg) (Figure 2B) In addition, double immunostaining and co-localization analysis for Iba1 and CD11b on cerebellar slices under similar conditions, demonstrated that the microglial activation took place mainly in the molecular layer of the cerebellum, rather than in the granular layer (Figure 2C). In this cerebellar layer, microglia acquired a bushy morphology according to the perimeter of the soma and the length of the branches after THC treatment cessation (Figure 2D). Notably, the microglial activation phenotype was not detectable in other brain areas where CB1R are heavily expressed such as hippocampus, frontal cortex or striatum (Supplemental Figure 4).

The quantitative analysis of mRNA for CB2R and the neuroinflammatory markers IL-1β, TNF-α, COX-2, CD11b, and CXCL2 indicated that the expression of these genes is enhanced in the cerebellum 5 d after the cessation of sub-chronic THC treatment at the dose of 5 and 20

mg/kg (Figure 2E).

In an additional experimental group, the cannabinoid withdrawal syndrome was precipitated after sub-chronic THC (20 mg/kg) treatment by rimonabant (10 mg/kg, i.p.) administered 4 h after the last THC injection(31). Control groups, sub-chronically treated with vehicle receiving an acute challenge of rimonabant or its vehicle, were also run in parallel. We found that under rimonabant-triggered cannabinoid withdrawal the results were similar on the reactivity of cerebellar microglia (Supplemental Figure 5, A and B) and the down-regulation of cerebellar CB1R (Supplemental Figure 5C) than those here reported after spontaneous cessation of sub-chronic THC treatment.

We hypothesized that the neuroinflammatory phenotype observed in the cerebellum molecular layer 5 d after THC cessation would respond to a possible restricted alteration in the extracellular milieu resulting from the de-regulated glutamate handling in parallel fiber terminals as a consequence of the CB1R down-regulation. As expected, a strong decrease in CB1R expression was also detected in the cerebellum at the end of the sub-chronic THC treatment (Supplemental Figure 6A), in agreement with previous studies(8). In contrast, no changes in CD11b expression were detected in cerebellar homogenates (Supplemental Figure 6A), although the microglial morphology was slightly altered (Supplemental Figure 6B). No signs of cellular death were revealed at the end of sub-chronic THC treatment in the cerebellum using the Fluoro-Jade B assay suggesting that microglial reactivity was not associated to cytotoxicity processes (Supplemental Figure 7, A and B). Moreover, no signs of microglia (Supplemental Figure 7, C and D) or astroglia (Supplemental Figure 7, E and F) proliferation were observed in the cerebellum at the end of this treatment as measured by cell counting and immunoblot analysis of Iba1 and glial fibrillary acidic protein (GFAP) expression. Similarly, the neuroinflammatory markers IL-1β, TNF-α, COX-2, CXCL2 and IL-10 were not altered in the cerebellum at the end of the THC treatment (Supplemental

Figure 7G).

When the time course of these effects mediated by THC exposure was studied, we observed that CD11b expression increased in the cerebellum only 5 d after THC cessation, while CB1R expression down-regulation reached the maximum level at the end of sub-chronic THC treatment, slowly recovering afterwards (Supplemental Figure 8A). The time course for the effect of THC exposure on microglial morphology revealed a progressive modification of these cells to the activated phenotype (Supplemental Figure 8B). In addition, acutely dissociated cerebellar cells positive (CD11b+, population 4, P4) or negative (CD11b–, population 5, P5) for CD11b, from mice receiving sub-chronic Veh, THC-5 or THC-20 and sacrificed 5 d after the end of treatment, were sorted. These cells were analyzed by RT-PCR for the mRNA expression of CB1R, CB2R and the pro-inflammatory cytokine IL1β (Figure 2F). Under these conditions, the percentage of CD11b+ (P4) cells slightly increased in the THC-20 group. In the CD11b+ (P4) population, mainly corresponding to cells positive for Iba-1 staining (Supplemental Figure 9), an enhanced expression of CB2R and IL-1β was revealed (Figure 2F). Instead, CD11b– (P5) cells did not present a modulation of CB2R and IL-1β gene expression, but showed an enhanced expression of CB1R mRNA (Figure 2F).

Therefore, the inflammatory phenotype in the cerebellum was progressively enhanced after sub-chronic THC cessation and correlated with the poor performance of cerebellar function.

Blockade of microglial activation ameliorates cerebellar deficits

We used the tetracycline antibiotic minocycline, an immunosuppressant with inhibitory properties on microglial activation(35), to evaluate the role of this process in the motor coordination deficit described above after the cessation of sub-chronic THC treatment. Minocycline (40 mg/kg, i.p.) was administered 1 h after the last THC injection (5 or 20 mg/kg, i.p.) on the sixth day of THC treatment, and once a day during 5 d. Minocycline sub-

chronic treatment reversed the enhancement of CD11b levels in the cerebellum promoted by sub-chronic THC administration (Figure 3A). Moreover, minocycline treatment normalized microglial morphology to control levels regarding both the perimeter of the soma and the length of the branches (Figure 3B). Cerebellar CB1R expression was not affected after minocycline administration, as detected by immunoblot of cerebellar samples (Figure 3C) and by immunofluorescence detection in the molecular layer of the cerebellum (Figure 3D).

The compromised conditioned cerebellar learning of THC-withdrawn mice was normalized after minocycline blockade of microglial activation. As above, the first conditioning session (C1) was performed on the last day of the sub-chronic THC (5 or 20 mg/kg) or vehicle treatment corresponding to the first day of minocycline (or saline) administration (C1, Figure 4A and 4C, Supplemental Figure 1B). As shown previously, the groups that had received sub-chronic THC showed a dose-dependent deficit in associative learning compared to control groups during the conditioning phase (Figure 4, A and C). The administration of minocycline increased the percentage of conditioned responses starting on the third day of treatment (C3, Figure 4, A and C). In another experimental set, THC-withdrawn mice were tested for motor coordination the fifth and last day of minocycline administration using the coat-hanger test. Minocycline also reversed the motor coordination impairment induced by THC, improving the coat-hanger test performance compared to sub-chronic THC mice receiving saline (Figure 4, B and D). Therefore, microglial activation blockade correlates with a reduction in the motor coordination deficits observed in spontaneous THC-withdrawn mice. In an additional experiment, we verified that acute minocycline administration did not modify the somatic manifestations of rimonabant (10 mg/kg, i.p.) mediated withdrawal syndrome after sub-chronic THC (20 mg/kg, i.p.) treatment, which discards a possible acute effect of minocycline on the withdrawal manifestations (Supplemental Figure 10).

Together, these results suggest the involvement of microglial activation on conditioned

cerebellar learning and motor coordination impairments promoted by sub-chronic THC administration, given that the optimal performance on both functional tests requires an intact cerebellar-mediated function.

CB1R down-regulation is critical for cerebellar neuroinflammation and function

To evaluate the relevance of CB1R down-regulation in the neuroinflammatory and behavioral alterations here reported, we investigated these responses in CB1R KO mice (*CB1*$^{-/-}$). Mild motor coordination deficits have been previously reported in *CB1*$^{-/-}$ adult mice (3-5 months) characterized by rotarod(36) and eye-blink conditioning test(17). We observed an increase in CD11b expression in cerebellum, but not in the hippocampus on naïve *CB1*$^{-/-}$ mice (Figure 5A). Moreover, the percentage of acutely dissociated cerebellar cells sorted as CD11b+ (P4) were more abundant in *CB1*$^{-/-}$ than in controls, and expressed enhanced mRNA levels of CB2R and IL-1β (Figure 5B).

The neuroinflammatory phenotype observed in the *CB1*$^{-/-}$ mice was also sensitive to minocycline treatment, since CD11b expression (Figure 5C) and the morphological changes highlighted by Iba1 staining of microglia (Figure 5D) were normalized in the cerebellum after minocycline sub-chronic administration. Moreover, an enhanced mRNA expression of CB2R and inflammatory mediators, such as IL-1β, IL-10, TNF-α, COX-2 and CXCL2, was detected in the cerebellum of *CB1*$^{-/-}$ mice, and these changes were sensitive to minocycline treatment (Figure 5E). Additionally, no changes in the expression of GFAP were observed in the cerebellum of *CB1*$^{-/-}$ mice (Supplemental Figure 11).

The analysis of cerebellar function after minocycline treatment in *CB1*$^{-/-}$ mice revealed the remarkable recovery of the disrupted response in conditioned cerebellar learning (Figure 5F; Supplemental Figure 12A). Similarly, the motor coordination impairment revealed in *CB1*$^{-/-}$

mice by the coat-hanger test was also significantly improved by minocycline treatment (Figure 5G), demonstrating the relevance of microglial activation in the phenotype promoted by CB1R deletion.

IL-1 receptor signaling blockade resolves the cannabinoid-mediated cerebellar deficits

As described above, cerebellar IL-1β expression was enhanced 5 d after sub-chronic THC treatment cessation in CD11b+ (P4) cells, and is constantly altered in *CB1*$^{-/-}$ mice. Local microinjection of IL-1β in the cerebellum is known to produce ataxia(37) and enhances the firing rates of cerebellar Purkinje neurons(38) acting on IL-1 receptors (IL-1R) strongly expressed in Purkinje neurons(39). We therefore tested whether an inhibitor of IL-1R signaling, an end protected L-valyl derivative with antagonistic properties on IL-1R (IL-1RA), would improve cerebellar performance under our experimental conditions. For delayed eyeblink conditioning analysis, mice received IL-1RA (100 mg/kg, i.p.) 4 d after sub-chronic THC treatment cessation during three consecutive days 4 h before the test session (C5-C7, Figure 6A; Supplemental Figure 1C). Mice receiving IL-1RA showed an increase in the number of conditioned responses that reached statistical significance from the second day of IL-1RA administration for THC (5 mg/kg) and from the first day of administration for THC (20 mg/kg) (Figure 6A). Similarly, *CB1*$^{-/-}$ mice improved transiently their performance in the delayed eyeblink conditioning paradigm after IL-1RA administration (Figure 6B; Supplemental Figure 12B). Correspondingly, the acute IL-1RA administration 4 h before the coat-hanger test normalized the performance of mice that received sub-chronic THC (5 or 20 mg/kg) to that of control mice (Figure 6C). A similar treatment with IL-1RA in *CB1*$^{-/-}$ mice significantly improved their performance in the coat-hanger test (Figure 6D). Interestingly, no effect of IL-1RA was observed in control groups (sub-chronic VEH (5 d) and WT mice),

either in the conditioned cerebellar learning test or the coat-hanger test (Figure 6). Moreover, no alteration of cerebellar microglia morphology was detected after acute IL-1RA administration (Supplemental Figure 13), pointing to a specific role of this blockade strategy under conditions of neuroinflammation.

Cerebellar neuroinflammation modulates Arc/Arg3.1 expression

The activity of cerebellar Purkinje neurons can be evaluated by measuring the expression of the activity-regulated cytoskeleton-associated protein, also known as Arg3.1 (Arc/Arg3.1)(40). Arc/Arg3.1 is expressed in Purkinje cell neurons where it colocalizes with calbindin immunoreactivity (Figure 7A). We measured Arc/Arg3.1 expression in cerebellar homogenates to assess the Purkinje cell activity under the experimental conditions studied above. Mice after sub-chronic THC treatment cessation (Figure 7B) and $CB1^{-/-}$ mice (Figure 7C) showed an enhanced expression of Arc/Arg3.1 compared to control mice. Interestingly, a clear reduction of Arc/Arg3.1 expression was observed after sub-chronic minocycline treatment in both experimental conditions (Figure 7, B and C).

Immunofluorescence analysis for Arc/Arg3.1 revealed the localized increase in the expression of Arc/Arg3.1 in the Purkinje cells of the cerebellum both after sub-chronic THC treatment cessation (Figure 7D) and in $CB1^{-/-}$ mice (Figure 7E). This result was corroborated by the intensity analysis performed across the different layers of the cerebellar cortex (Figure 7, D and E). These results point to a relevant involvement of the neuroinflammation process produced by the down-regulation of cerebellar CB1R in the activity-related changes (expression of Arc/Arg3.1) promoted in Purkinje cells.

CB1R de-regulation in parallel fibers recreates the cerebellar deficit

To better evaluate whether the down-regulation of CB1R in the cerebellar parallel fibers would affect microglial responses and the cerebellar performance, we used a conditional mouse model lacking CB1R in cerebellar granular cells, the *CB1α6⁻* mice(12). In this mouse line, CB1R were absent from the parallel fibers, but they were preserved in other cerebellar locations where CB1R are less abundantly expressed, such as the climbing fibers (Supplemental Figure 14). In this mouse line, CB1R detection by immunofluorescence on microglial cells located in the molecular layer of the cerebellum was negligible and indistinguishable from background staining (Supplemental Figure 15). Microglial morphology revealed by staining with Iba1 showed an activated phenotype (Figure 8A) of these cells in the cerebellar molecular layer of the *CB1α6⁻* mice. These mice displayed an enhanced expression of CB2R, IL-1β and TNF-α in cerebellar mRNA (Figure 8B). The increased expression of CB2R and IL-1β was restricted to CD11b+ (P4) cells, but not detected in CD11b– (P5) cells acutely isolated from the cerebellum (Figure 8C). Similar to previous data with *CB1⁻/⁻* mice, *CB1α6⁻* mice showed an enhanced expression of cerebellar Arc/Arg3.1 (Figure 8, D and E), and exhibited a significant motor coordination deficit in the coat-hanger test compared to their control littermates (Figure 8F), that was prevented by acute IL-1RA administration (Figure 8G). The use of this novel genetic tool provides direct evidence of the role of CB1R in cerebellar parallel fibers in the modulation of these morphological and behavioral responses.

Discussion

Our study describes the local activation of cerebellar microglia as a result of dysregulated CB1R activity produced by both sub-chronic THC exposure and CB1R genetic disruption, which correlates with functional deficits on cerebellar conditioned learning and motor coordination. The microglial activation was typified by alterations in microglial morphology, mainly in the molecular layer of the cerebellum, and was accompanied by the enhanced expression of specific pro-inflammatory genes, such as IL-1β. Pharmacological blockade of microglial activation and IL-1R signaling prevented the deficit in cerebellar associative learning and motor coordination in sub-chronically THC-treated mice and in $CB1^{-/-}$ mice, Altogether, these results reveal the critical role of microglial mediated signaling in the cerebellar dysfunctions associated to CB1R de-regulation.

Microglial cells play an important role in innate immune responses in the CNS(21,24). Under pathological conditions, microglial cells are activated through complex processes that produce remarkable changes in their morphology and gene expression(26,29,30). Cessation of sub-chronic THC treatment and genetic disruption of CB1R produced selectively in the cerebellum an increased expression of CD11b protein, a marker of activated microglia, enhanced expression of pro-inflammatory cytokines and alteration of microglial morphology mainly in the cerebellar molecular layer. Sorted CD11b+ (P4) cerebellar cells from these mice showed an increased expression of CB2R and IL-1β mRNA, an effect not observed in CD11b– (P5) cells. Under these experimental conditions, CB1R expression was enhanced specifically in the CD11b– (P5) cells but not on CD11b+ (P4) cells. This result correlates with that obtained for CB1R mRNA expression under similar THC treatment conditions in rat cerebellum(41). This microglial activation was not associated to cell stress as revealed by the Fluoro-Jade B assay. Microglial cells in resting conditions present low to undetectable levels of CB1R and CB2R mRNA and protein(20). Therefore, the microglial activation process

triggered by THC is unlikely to be mediated by the direct effect of THC on the brain microglial population, and it is likely related to the de-regulation of CB1R function specifically in the cerebellum. In contrast with CB1R protein down-regulation, cerebellar CB2R mRNA was up-regulated in parallel to the development of the neuroinflammatory phenotype in the CD11b+ cells. It is plausible that THC, acting as an agonist on microglial CB2R, may reduce microglial activation through CB2R modulation(42,43) during exposure to THC. After THC cessation, microglia would be released from the CB2R-mediated inhibition of this cannabinoid agonist. Under these conditions, microglia would remain activated while CB1R functionality is recovered. In agreement with the selectivity of this response, CB1R genetic removal was sufficient to induce a similar change in microglial phenotype in the cerebellum, but not in the hippocampus. In this regard, a previous study reported that 12 months old $CB1^{-/-}$ mice, but not 2 or 5 months old $CB1^{-/-}$ mice displayed enhanced neuroinflammation in the hippocampus(44). The microglial activation reported herein in the cerebellum of 2-3 months old constitutive $CB1^{-/-}$ and $CB1\alpha6^{-}$ mice reveals the particular sensitivity of this brain area to the neuroinflammatory process promoted by the down-regulation of CB1R activity.

We evaluated the relationship between the microglial activation and the cerebellar deficits using minocycline, a second-generation tetracycline antibiotic with inhibitory properties on microglial activation(35), probably due to its blocking properties on 5-lipoxigenase expression(45). Minocycline significantly reduced the cerebellar conditioned learning and motor coordination deficits in THC withdrawn and $CB1^{-/-}$ mice together with the landmarks of microglial activation. Notably, this improvement in cerebellar function was not associated to a recovery of the CB1R density in the cerebellum of mice after sub-chronic THC treatment cessation. Moreover, minocycline was effective at improving cerebellar conditioned learning and motor coordination in mice lacking CB1R pointing to a so far unknown role for the

microglial reactivity status in the cerebellar performance.

Microglial activation was associated to enhanced expression of CB2R and some particular pro-inflammatory factors, such as IL-1β, in the cerebellum of mice sub-chronically treated with THC and in *CB1*$^{-/-}$ mice. This result was supported by the enhanced expression of CB2R and IL-1β in CD11b+ cells, but not in CD11b– cells sorted after cerebellar cell dissociation in THC exposed mice and mice with genetic disruptions on CB1R expression. IL-1β is mainly produced in the brain by microglia(46) and it has been reported to directly regulate Purkinje cell activity(38). IL-1RA administration induced a recovery on the cerebellar conditioned learning as well as on motor coordination in THC-withdrawn and *CB1*$^{-/-}$ mice. In this mouse line, both minocycline and IL-1RA significantly improved the performance in cerebellar conditioned learning and motor coordination, a result that reveals the crucial implication of microglial activation on cerebellar functionality. Therefore, the neuroinflammation produced as a consequence of the CB1R down-regulation would be a key factor in the cerebellar functional deficits reported previously(36,17) and herein.

The cerebellar conditioned responses recorded on the eyeblink conditioning test for THC-withdrawn mice and *CB1*$^{-/-}$ mice that received IL-1RA treatment were markedly different on the last conditioning days. Indeed, IL-1RA treatment produced a transitory improvement of conditioned responses in *CB1*$^{-/-}$ mice only during treatment, whereas the improvement on conditioned responses in THC-withdrawn mice was observed even beyond the IL-1RA treatment. This result reveals the recovery of CB1R functionality on THC-withdrawn mice at the end of the conditioning period compared with *CB1*$^{-/-}$ mice, where CB1R are permanently eliminated. IL-1RA administration had immediate effects on the performance of cerebellar conditioned learning, and these effects were more relevant in those conditions where microglial activation was markedly strong, such as the high dose of THC (20 mg/kg) or the *CB1*$^{-/-}$ mice. Instead, the effects of minocycline do not appear after a single administration,

and were only revealed after the second-third day of treatment. This is in agreement with the idea that IL-1β would be the effector produced by activated microglia and involved in the alteration of cerebellar function. Instead, minocycline would be associated with a switch in the microglial activation status to a non-reactive state where IL-1β expression would be reduced. Notably, the direct action of IL-1β on Purkinje cells has been associated to an increase on cellular excitability that could directly affect cerebellar output(38). The enhanced activity of Purkinje neurons under neuroinflammatory conditions fits with the increased expression of cerebellar Arc/Arg3.1 protein reported herein, and proposed previously(40). The increase in Purkinje neuron activity may affect the functionality of their projecting area leading to deficits in specific cerebellar functions.

A possible mechanism to explain this neuroinflammatory process would be the putative glutamate mishandling from CB1R-containing de-regulated terminals, since extracellular glutamate concentrations are tightly regulated at synapses onto Purkinje cells(47). Indeed, the excitatory transmission to Purkinje cells shows an extended presence of glutamate in the synaptic cleft(48). Moreover, the endocannabinoid system is strongly activated in the case of glutamate spillover at this particular synapse to rapidly shut-down glutamate release(49). In agreement, an increased postsynaptic excitatory activity has been reported in cerebellar Purkinje cells after sub-chronic THC-mediated CB1R down-regulation(14). In addition, different forms of synaptic plasticity between parallel fibers and Purkinje neurons mediated by glutamate are suppressed in $CB1^{-/-}$(11) and $CB1\alpha6^{-}$ mice(12).

Our results suggest that the neuronal/glial circuitry in the cerebellum is particularly sensitive to conditions affecting CB1R functionality. In this regard, it was shown that chronic cannabis consumers assessed during periods of abstinence demonstrate hypoactive cerebellar activity(50), which could be interpreted as a consequence of CB1R down-regulation in the cerebellar cortex. Indeed, chronic cannabis consumption in humans produced a clear down-

regulation in brain CB1R mRNA levels as determined via post-mortem [^{3}H]-rimonabant binding(9). These results uncover a new neurobiological mechanism for the deleterious effects of THC on cerebellar function that may underlie the recent observations in the cerebellar motor learning acquisition in current(15) and former(18) cannabis users. The neuronal circuits involved in the eyeblink conditioning response are the same in mouse and human, thereby demonstrating the translational relevance of this test.

Therefore, we hypothesize (Supplemental Figure 16) that these cerebellar neuroinflammatory alterations, as a consequence of CB1R down-regulation, represent the neurobiological basis for this side-effect related to cannabis consumption. Interestingly, a more difficult-to-acquire learning paradigm, the trace eyeblink conditioning, which requires forebrain function, was not affected in cannabis users(19), pointing to the specific disruption promoted by repeated THC exposure in this form of cerebellar learning. The reversal of these cerebellar deficits in our animal models by the blockade of microglial activation and IL-1RA opens new interesting therapeutic approaches for these side effects promoted by cannabis use.

Methods

Animals

Male mice aged between 7 and 10 weeks were used. Wild-type Swiss albino mice (CD-1) were purchased from Charles River; *CB1*$^{-/-}$ mice in CD-1 background(51), and *CB1a6*$^{-}$ in C57BL/6 background(12) were bred at our animal facility. Both littermate- and age-matched controls were used and found to be indistinguishable; data for controls were thus pooled. *Gabra6Cre* and *CB1f/f* animals were maintained on a C57BL/6-J background as described previously(52). *Gabra6cre;CB1f/f* males were mated with *CB1f/f* females to generate *Gabra6cre;CB1f/f* experimental animals and *CB1f/f* littermate controls. After arrival to the facility mice were housed in plastic cages of four and maintained at a controlled temperature (21 ⊥ 1 °C) and humidity (55 ⊥ 10 %) environment. Food and water were available *ad libitum*. Lighting was maintained at 12-h cycles (on at 8 am and off at 8 pm). All the experiments were performed during the light phase of the dark/light cycle. The animals were habituated to the experimental room and handled for 1 week before the start of the experiments. All behavioral experiments were performed under experimental conditions blind to the observer.

Drugs and treatments

Delta9-tetrahydrocannabinol (THC) was purchased from THC Pharm GmbH; cremophor-EL and minocycline (MIN) were purchased from Sigma; rimonabant (RIM) was generously provided by Sanofi-Aventis Recherche and IL-1R Antagonist (IL-1RA) was purchased from Merck Millipore. THC and RIM were diluted in vehicle (VEH) solution (5 % ethanol: 5 % cremophor-EL: 90 % saline). MIN was dissolved in 0.9 %

saline (SAL). IL-1RA was dissolved in DMSO. THC, RIM, MIN and their vehicles were administered i.p. in a volume of 0.1 mL/10 g of body weight. IL-1RA and its vehicle (DMSO) were administered i.p. in 0.02 mL/10 g of body weight. Sub-chronic THC (or VEH as a control) was administered following a protocol that produced cannabinoid physical dependence(31). Thus, mice were injected with THC (1 mg/kg, 2.5 mg/kg, 5 mg/kg or 20 mg/kg, i.p.) or VEH twice daily at 09:00 and 19:00 h during five consecutive days. On the sixth day, mice received only the morning injection of THC or VEH. Mice were sacrificed 4 h after this last injection (sub-chronic THC-5, sub-chronic THC-20 and sub-chronic VEH groups). To study the effects of the spontaneous cannabinoid withdrawal mice were analyzed 5 d after the end of the cannabinoid or vehicle treatment: sub-chronic THC-1 (5 d), sub-chronic THC-2.5 (5 d), sub-chronic THC-5 (5 d), sub-chronic THC-20 (5 d) and sub-chronic VEH (5 d) groups.

To evaluate the precipitated THC withdrawal, mice received RIM (10 mg/kg, i.p.) 4 h after the last THC (sub-chronic THC+RIM (5 d) group) or VEH (sub-chronic VEH+VEH (5 d) group) administration. Mice were sacrificed 5 d later.

To assess the role of microglial activation in the cerebellar function after the spontaneous withdrawal of sub-chronic THC exposure, mice were first treated sub-chronically with THC (5 mg/kg or 20 mg/kg) or VEH. On the sixth day, and 3 h after the last VEH or THC injection, mice received the first administration of MIN (40 mg/kg, i.p., once per day, 5 d). Experimental groups were: sub-chronic VEH+MIN (5 d) and sub-chronic VEH+SAL (5 d); sub-chronic THC-5+MIN (5 d) and sub-chronic THC-5+SAL (5 d); sub-chronic THC-20+MIN (5 d) and sub-chronic THC-20+SAL (5 d). Similarly, $CB1^{-/-}$ mice and WT controls were treated during 5 d with MIN (40 mg/kg, i.p., once per day). Experimental groups were: WT+SAL and WT+MIN; $CB1^{-/-}$

+SAL and *CB1*$^{-/-}$+MIN. Mice were analyzed for conditioned cerebellar learning as it is described in Supplemental Figure 1B and Supplemental Figure 12A, and for motor coordination at the end of the MIN sub-chronic treatment. Afterwards, mice were sacrificed for biochemical or immunohistochemical assays.

To study the effect of IL-1RA (100 mg/kg, i.p.) in conditioned cerebellar learning, mice were treated sub-chronically with VEH, THC-5 and THC-20 (during 5 d, twice per day and once on the sixth day). Four days after the last VEH, THC-5 or THC-20 injection they received an acute administration of IL-1RA or DMSO (see Supplemental Figure 1C). In addition, *CB1*$^{-/-}$ mice and WT controls were also studied for the effects of IL-1RA in cerebelar conditioned learning (see Supplemental Figure 12B). Four hours after IL-1RA administration mice were assayed in the eyeblink conditioning paradigm repeating this procedure for three consecutive days (sessions C5 to C7). In another set of animals, IL-1RA or DMSO were injected acutely to THC-withdrawn mice, *CB1*$^{-/-}$, *CB1a6*$^{-}$ and control mice to study motor coordination in the coat-hanger test. In this case mice were subsequently sacrificed for biochemical and immunohistochemical analysis.

Immunoblot analysis

Frozen cerebellar, striatal, cortical or hippocampal tissues were processed as previously reported(53). For immunoblotting, we used the following antibodies: anti-CD11b (rabbit, 1:500) from AbCam; anti-Arc/Arg3.1 (mouse, 1:100) and anti-GAPDH (mouse, 1:5,000) from Santa Cruz Biotechnology; anti-CB1R (rabbit, 1:1,000) from Frontier Science; anti-Iba1 (rabbit, 1:500) from Wako Pure Chemical Industries and anti-glial

fibrillary acidic protein (GFAP) (rabbit, 1:500) from Dako. Blots containing equal amounts of cerebellar protein samples (40 µg/lane) were probed with different primary antibodies. Bound primary antibodies were detected with horseradish peroxidase–conjugated antibodies to mouse (Thermo Fisher Scientific) (diluted at 1:5,000) and to rabbit (Cell Signaling Technologies) (diluted at 1:10,000). Both antibodies were visualized by enhanced chemiluminescence detection (West-Femto-SuperSignal, Thermo Fisher Scientific). When necessary, Immobilon-P membranes (Millipore) were stripped before re-blocking and re-probing. The optical density of the relevant immunoreactive bands was quantified after acquisition on a ChemiDoc XRS System (Bio-Rad) controlled by The Quantity One software v4.6.3 (Bio-Rad). The quantification of protein expression was in the linear range for the experimental conditions used in this study (data not shown). For quantitative purposes, the optical density values for the proteins of interest were normalized to the detection of the house-keeping control GAPDH in the same samples and expressed as percentage of control treatment.

Tissue preparation for immunohistochemistry and immunofluorescence

After pharmacological treatment and/or behavioral testing, mice were deeply anesthetized by i.p. injection (0.2 ml/10 g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) prior to intracardiac perfusion of 4 % PFA in 0.1 M Na_2HPO_4/NaH_2PO_4 buffer, pH 7.5, delivered with a peristaltic pump at 19 ml/min for 5 min. Brains were removed and postfixed overnight at 4 ºC in the same fixative solution. Brain sections (30 µm) were obtained with a vibratome (Leica) and kept in a solution

containing 30 % ethylene glycol, 30 % glycerol and 0.1 M PB at −20 °C until processed for immunohistochemistry or immunofluorescence.

Immunohistochemistry

The immunohistochemical procedure was performed as previously described(54). The antibody for GFAP was used at 1:500 dilution in this procedure.

Immunofluorescence

Free-floating slices were rinsed in TBS (0.25 M Tris, 0.15 M NaCl, pH 7.5), incubated for 15 min in 0.2 % Triton X-100 in TBS and then incubated overnight at 4 °C with primary antibodies: anti-Iba1 (1:500), anti-CD11b (1:50) (Cederlane Laboratories Limited), anti-CB1R (1:1,000), anti-calbindin (1:300) and anti-Arc/Arg3.1 (1:100), both from Santa Cruz Biotechnology, and the double staining with anti-CB1R (rabbit, 1:1,000) from Frontier Science and anti-VGLUT1 (mouse, 1:500) from Merck Millipore. The next day (16 h later), after two rinses in TBS, sections were incubated for 2 h at room temperature with fluorescent anti-rabbit-Cy3 (1:500) (Jackson ImmunoResearch Laboratories) or biotinylated anti-mouse antibody (1:400, Vector Laboratories). After a 10-min wash, the slices were incubated for 15 min with streptavidin–Alexa Fluor 488 (Invitrogen). After two washes (10 min each), tissue sections were mounted onto gelatin-coated slides with Mowiol mounting media.

Confocal images were obtained using a Leica SP2 confocal microscope, adapted to an inverted Leica DM IRBE microscope. Alexa Fluor 488 and Cy3 were excited with the

488 nm line of an argon laser and the 543 nm line of a green neon laser, respectively. For double immunofluorescence images were taken for each animal in a sequential mode. All further image analysis was performed by an experimented observer blind to the experimental conditions.

CD11b+ Iba1+ microglia quantification: Stained sections were analyzed by confocal microscopy. We used the specific tool "Particle analysis, cell counter" from ImageJ software (NIH) for the manual quantification of CD11b+ Iba1+ positive microglial cells. 20-25 images from 4-5 animals (5 images per animal) were analyzed per each experimental group. We used this method to determine the number of Iba1+ and Iba1+ CD11b+ microglial cells after the spontaneous and precipitated THC withdrawal.

CD11b / Iba1 co-localization analysis: Confocal images were taken for each animal (n=5) in a sequential mode. A constant region of interest (ROI) surrounding the microglial cells (n=3 mice per group; 5 images per mouse) large enough to encompass all the cell ramifications with the cell body in the center (similar to that shown in Supplemental Figure 17), was determined. Afterwards, the Intensity Correlation Quotient (ICQ) between CD11b and Iba1 signal within each ROI was calculated with the ImageJ software.

CB1R intensity analysis: Confocal images (4 images per animal corresponding to the molecular and the granular layers of the cerebellum) were acquired (n=4-5 mice; 4-5 images per mouse) in a sequential mode. Afterwards, CB1R signal intensity was evaluated using a constant ROI with the ImageJ software.

Morphological analysis: To evaluate the changes in microglial morphology, confocal microscopy images of the whole microglial cells stained with Iba1 were acquired with

an oil immersion lens (40X) and a 1.5 zoom. Images were taken at different z levels (0.8 µm depth intervals) to evaluate the morphology of the whole cell. Afterwards, the length of the microglial ramifications and the perimeter of the microglial soma were analyzed with the ImageJ software (see Supplemental Figure 17 for details). In all cases, the number of microglial cells analyzed were 16-24 per group (*n*–4-5 mice; 4 cells per animal).

Arc/Arg3.1 intensity representation: To evaluate the Arc/Arg3.1 expression in the cerebellum, confocal images were analyzed using the ImageJ software (Plot Profile tool) for Arc/Arg3.1 signal intensity (gray value) along 300 µm. For this purpose, a line (300 µm) was drawn through the three cerebellar layers: granular layer (gl), Purkinje cell layer (PC), and molecular layer (ml) and the intensity of each pixels on that line was represented.

Fluoro-Jade B assay

Cell stress was evaluated with the Fluoro-Jade B assay (Chemicon) following manufacturer's instructions. In order to determine if cell death is accompanied by a microglial activation process, Iba1 immunofluorescence was performed followed by the Fluoro-Jade B assay.

Dissociation of cerebellar cells and CD11b-based cell sorting

Cerebellar tissue from vehicle (5 d), THC-5 (5 d) and THC-20 (5 d) groups, as well as *CB1*$^{-/-}$, WT, CB1α6^{-} and control mice (*n*=3-4 per group) were removed after anesthesia

and 3 min perfusion with ice cold 0.1 M PB, 0.9% NaCl. Tissues were enzymatically digested with Neuronal Tissue Dissociation Kit (Papain) (Miltenyi Biotec) following manufacturer's instructions. Cell suspension in HBSS was stained with PE-conjugated anti-CD11b (Miltenyi Biotec) and submitted to cell sorting to isolate the CD11b+ (P4) and CD11b– (P5) population. CD11b+ (P4) cells were mainly positive for Iba1 staining (Supplemental Figure 8). Both the CD11b+ (P4) and CD11b– (P5) fractions were collected and used for further analyses of gene expression by RT-PCR.

RNA extraction and reverse transcription

Cerebellar tissues and CD11b+ (P4) and CD11b– (P5) sorted cells were collected and stored at -80ºC. Isolation of total RNA was performed using the RNeasy Mini Kit (tissue) (Qiagen® GmbH) or the RNeasy Micro Kit (dissociated cells) (Qiagen® GmbH) following the manufacturer's instructions. The quality of the total RNA was assessed by the spectrophotometric ratio A260/A280 (1.9 to 2.1). The total RNA concentration was measured using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific).

Reverse transcription was performed with 0.3 µg of total RNA from each animal to produce cDNA in a 10 µl reaction with 200 units of SuperScript III Reverse Transcriptase (Invitrogen) and 500 ng oligo(dT)15 primers. Reverse transcriptase reactions were carried out at 25°C for 10 min, then 50 min at 42°C and 15 min at 70°C. The cDNAs from cerebellar tissues were diluted 1:4 and stored at -20ºC until used, while cDNAs from CD11b+ (P4) and CD11b– (P5) cells were stored without further dilution until used.

Quantitative real-time PCR analysis

Real-time PCR was carried out with ABI PRISM® 7700 Sequence Detection System (PE Applied Biosystems), using the SYBR Green PCR Master Mix (PE Applied Biosystems) according to the manufacturer's protocol. All the samples were tested in triplicate and the relative expression values were normalized to the expression value of GAPDH.

Primers specific for mouse CB2R (sense, 5'-ggtcgactccaacgctatcttc-3'; antisense, 5'-gtagcggtcaacagcggttag-3'), IL-1β (sense, 5'-gaagagcccatcctctgtgact-3'; antisense, 5'-gttgttcatctcggagcctgtag-3'), TNF-α (sense, 5'-gactagccaggagggagaacag-3'; antisense, 5'-cagtgagtgaaagggacagaacct-3'), COX-2 (sense, 5'-ggccgactaaatcaagcaaca-3'; antisense, 5'- caatgggcataaagctatggttaga-3'), CXCL2 (sense, 5'-actgcatctgccctaaggtctt-3'; antisense, 5'-tgcttgaggtggttgtggaa-3'), CD11b (sense, 5'- ggatccggaaagtagtgagagaac-3' ; antisense, 5'- ccgaatttttctccatctgtgat-3'), IL-10 (sense, 5'- ggcgctgtcatcgatttctc-3' ; antisense, 5'- gccttgtagacaccttggtcttg-3') and GAPDH (sense, 5'-atgactccactcacggcaaat-3', antisense, 5'-gggtctcgctcctggaagat-3'), used as an endogenous housekeeping control to standardize the amount of target cDNA. Amplified PCR products were separated on a 2% agarose gel and stained with ethidium bromide to confirm the specificity of the primers.

Samples were analyzed by the double delta Ct (ΔΔCt) method. ΔΔCt values were calculated as the ΔCt of each test sample (different pharmacological treatments) minus the mean ΔCt of the calibrator samples (vehicle-saline group) for all the genes analyzed. The fold change was calculated using the equation $2^{(-\Delta\Delta Ct)}$.

Somatic expression of THC withdrawal

The somatic signs of THC withdrawal were precipitated 4 h after the last THC or vehicle injection(31). Briefly, mice were placed in a circular clear plastic observation area (30 mm diameter x 35 mm height) for a 15 min period of habituation. Immediately after habituation animals were observed for a further period of 15 min, followed by administration of rimonabant (10 mg/kg, i.p.). Then, mice were observed for a 45 min period. Observations of somatic signs before and after rimonabant challenge were divided in 5 min time intervals. A global withdrawal score was then inferred(31).

Delayed eyeblink conditioning

Mice assigned for classical conditioning of eyelid responses were prepared as follows. Firstly, they were anesthetized with 0.8–1.5 % isoflurane, supplied from a calibrated Fluotec 5 (Fluotec-Ohmeda) vaporizer, at a flow rate of 1–4 L/min oxygen (AstraZeneca) and delivered by a mouse anesthesia mask (David Kopf Instruments). Once anesthetized, animals were implanted with bipolar recording electrodes in the left orbicularis oculi muscle and with stimulating electrodes on the ipsilateral supraorbital nerve. Electrodes were made of 50 μm, Teflon-coated, annealed stainless steel wire (A-M Systems). Electrode tips were bared of the isolating cover for 0.5 mm and bent as a hook to allow a stable insertion in the upper eyelid. A bare silver wire affixed to the skull served as a ground. All the implanted wires were soldered to two four-pin sockets (RS Amidata). Bone screws and dental cement fixed the cannula and the sockets to the skull.

After the implantation of the stimulating and recording electrodes, mice were randomly assigned to the experimental groups (see Supplemental Figure 1, and Supplemental Figure 12 for detailed chronogram). All experimental groups WT, *CB1*$^{-/-}$, VEH or THC-1/2.5/5/20 were familiarized to the experimental chambers for 30 min during three consecutive days. During this period, the intensity thresholds for the activation of the supraorbital nerve and the proper implantation of recording electrodes in the orbicularis oculi muscle were checked. Then, mice were habituated to the tone (conditioned stimulus) for two consecutive days. Afterwards, the conditioning sessions were performed once daily for nine consecutive days starting the next day after the second habituation day (H2). The first conditioning session was performed on the sixth day of the THC treatment matching with the first day of MIN (or SAL) (Supplemental Figure 1B). In the case of the *CB1*$^{-/-}$ and WT mice, the conditioning sessions and the SAL or MIN administration, were performed following the schedule described in the Supplemental Figure 12A. Conditioning sessions started 1 h after MIN (or SAL) administration.

IL-1RA (100 mg/kg, i.p.) or its vehicle (DMSO), were administered on three consecutive days (C5, C6, C7), 4 d after the end of the cannabinoid sub-chronic treatment (Supplemental Figure 1C and Supplemental Figure 12B. Conditioned sessions started 4 h after the IL-1RA or DMSO administration.

The percentage of conditioned responses was computed everyday during 9 d. Experimental sessions were carried out with four animals at a time. Animals were placed in separate small (5×5×10 cm) plastic chambers located inside a larger (30×30×20 cm) Faraday box. The electromyographic activity of the orbicularis oculi

muscle was recorded with Grass P511 differential amplifiers (Grass-Telefactor), at a bandwidth of 0.1 Hz-10 kHz.

Classical eyeblink conditioning was achieved by the use of a delay-conditioning paradigm. A tone (370 ms, 600 Hz, 90 dB) was used as conditioned stimulus (CS). The tone was followed 270 ms from its onset by a 500 μs, 3× threshold, square, cathodal pulse applied to the supraorbital nerve. Thus, the tone and the pulse terminated simultaneously. A conditioning session consisted of 60 CS-US presentations, and lasted approximately 30 min. For a proper analysis of conditioned responses, the CS was presented alone in 10 % of the cases. CS-US presentations were separated at random by 30 ± 5 s. For habituation sessions, only the CS was presented, also for 60 times per session, at intervals of 30 ± 5 s. As criterion, we considered a "conditioned response" the presence, during the CS-US interval, of electromyographic activity lasting >20 ms and initiated >50 ms after CS onset.

Motor coordination tests

Rod test: The equilibrium test consists of a horizontal steel rod (diameter: 1 cm, length: 50 cm) suspended 40 cm height from cushioned floor. Mice were placed in the middle of the horizontal rod and released when all four paws gripped it, in order to ensure a stable starting position. This test evaluates latency to fall (s) and skills mice have to hold 20 s upon the rod and walk from the central region to the edge (score 0: mouse falls down before 20 s; score 1: mouse holds 20 s upon the rod in the central region; score 2: mouse holds 20 s upon the rod and moves from the central region of the rod). This test was performed 2 times, with an interval of 2-4 min between trials.

Coat-Hanger test: A steel coat-hanger (diameter: 2 mm, length: 40 cm) divided into twelve segments (length: 5 cm) and suspended at a height of 35 cm from a cushioned surface was used. Mice were placed in the middle of the hanger and the behavior was evaluated for a total of 60 s. Fall latency, number of movements in 60 s and extreme latency (from the central region of the hanger to the edge) were recorded. The test finished when the animal fell down or when the 60 s elapsed.

Rotarod test: The accelerating rotarod (five-lane accelerating rotarod; LE 8200, Panlab) was used to measure motor balance and coordination 5 d after the spontaneous cannabinoid withdrawal. During two consecutive days before the test day, mice were trained to hold at a constant speed (4 rpm) for at least 60 s. On the test day, the rod accelerated from 4 to 40 rpm in 5 min and the latency to fall was measured on ten consecutive trials. Data are expressed as the latency to fall on the first trial (Trial 1) and on the last trial (Trial 10).

Statistical analysis

Data are presented as mean ± SEM. The statistical significance was assessed by ANOVA, followed by a posteriori Post-hoc Dunnet's multiple comparison test when appropriate. P values <0.05 were considered significant.

Study approval

All animal procedures were conducted in accordance with the standard ethical guidelines (European Communities Directive 86/60-EEC) and approved by the local ethical committee (Comitè Ètic d'Experimentació Animal, CEEA-PRBB). Animal Welfare Assurance (#A5388-

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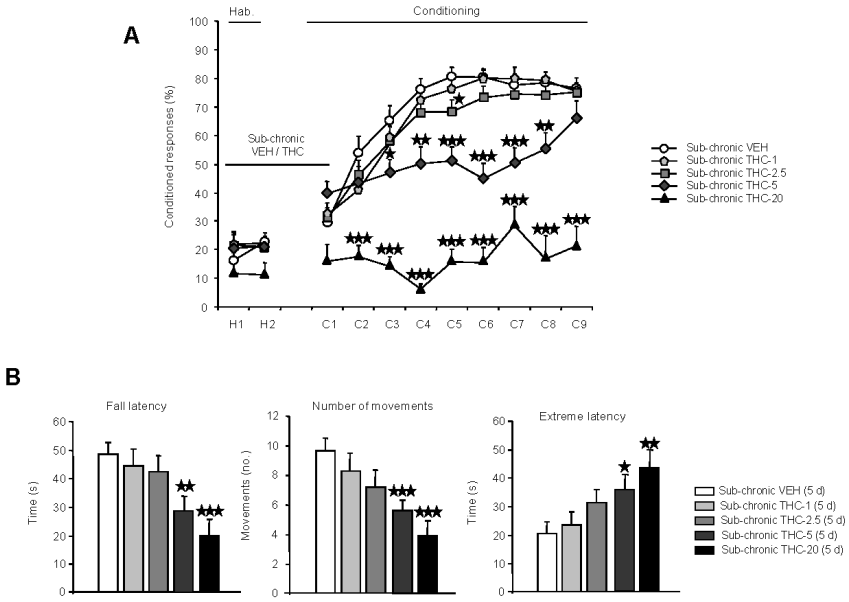

Figure 1. Cerebellar performance after sub-chronic THC exposure in mice. **(A)** Percentage of conditioned eyelid responses collected from mice treated with sub-chronic THC (1, 2.5, 5 and 20 mg/kg) or sub-chronic VEH (*n*=9-12 mice per group) (see Supplemental Figure 1A for experimental chronogram). **(B)** Motor coordination analysis in the coat-hanger test after exposure to sub-chronic THC (1, 2.5, 5 and 20 mg/kg) or sub-chronic VEH conditions 5 d after spontaneous withdrawal (*n*=11-17 mice per group). Impaired motor coordination was revealed by the fall latency, the number of movements to reach the end of the coat-hanger and the extreme latency. ★P<0.05, ★★P<0.01, ★★★P<0.001 vs. sub-chronic VEH.

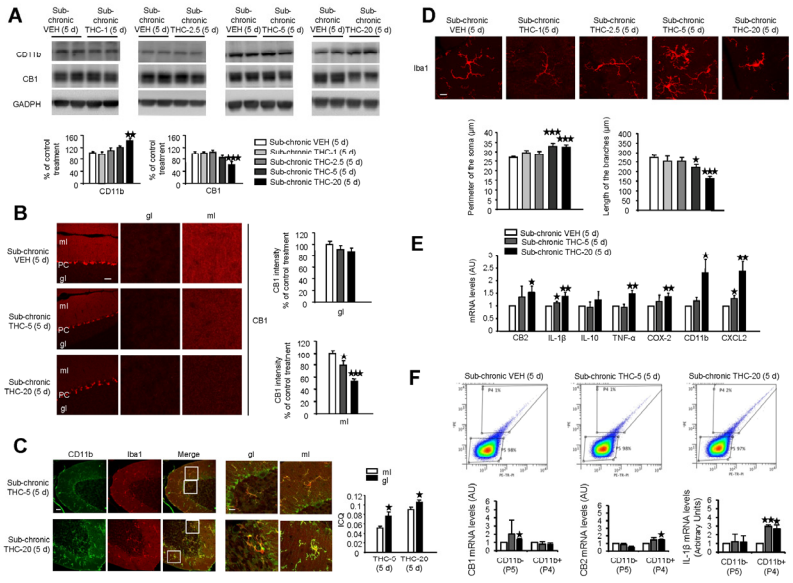

Figure 2. Characterization of cerebellar responses 5 d after THC treatment cessation. **(A)** Immunoblot and quantification of CD11b and CB1R in cerebellar homogenates from mice processed 5 d after receiving sub-chronically THC-1, THC-2.5, THC-5 and THC-20 or VEH (*n*=5-6 mice per group). The optical density for CD11b and CB1R was referred to GAPDH in the same samples. **(B)** Immunofluorescence and quantification of CB1R intensity in the granular and molecular layer of the cerebellum from mice processed 5 d after receiving sub-chronically THC-5 and THC-20 or VEH (*n*=3 mice per group, 5 images per mouse). ml: molecular layer, PC: Purkinje cell layer, gl: granular layer. Scale bar: 75µm. **(C)** Immunolocalization and intensity correlation quotient (ICQ) of Iba1 (red) and CD11b (green) in the molecular and granular layer of the cerebellum from mice treated sub-chronically with THC-5, THC-20, or VEH (*n*=3 mice per group, 3 images per mouse). Scale bar: 100µm. **(D)** Morphological analysis of Iba1+ microglial cells in the molecular layer of the cerebellum (*n*=4 mice per group, 4 cells per mouse). The perimeter of the cell soma and the length of the microglial branches from Iba1-stained cells were analyzed with the ImageJ software (see Supplemental Figure 17). Scale bar: 25µm. **(E)** mRNA expression analysis of CB2R and inflammation related genes in cerebellar samples (*n*=7-8 mice per group). (**F**) Flow cytometric analysis of CD11b expression and quantitative RT-PCR analysis on acutely dissociated cerebellar cells from VEH, THC-5 and THC-20 treated mice (*n*=3 per group). Sorted CD11b+ population (P4) and CD11b– population (P5) on THC-treated mice showed a differential expression of CB1R, CB2R and IL-1β. ★P<0.05, ★★P<0.01, ★★★P<0.001 vs. sub-chronic VEH+SAL (5 d).

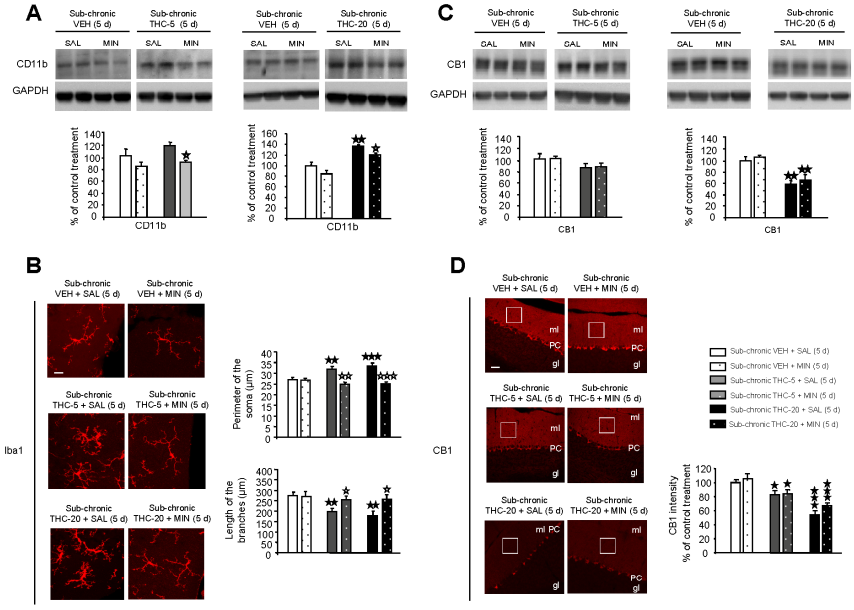

Figure 3. Minocycline administration after sub-chronic THC exposure prevents the activation of microglia in the cerebellum as well as the motor coordination impairment. **(A)** Immunoblot detection and quantification of cerebellar CD11b (*n*=5 mice per group) at the end of a sub-chronic exposure to minocycline (MIN) or saline (SAL) on sub-chronic THC (5 and 20 mg/kg) and sub-chronic VEH treatment conditions. **(B)** Morphological analysis on Iba1+ cells in cerebellar cortex (*n*=3-4 mice per group, 4-5 cells per mouse). Scale bar: 25 µm **(C)** Immunoblot detection and quantification of cerebellar CB1R (*n*=6 mice per group) at the end of MIN or SAL exposure on sub-chronic THC (5 and 20 mg/kg) and sub-chronic VEH treatment conditions. **(D)** Immunolocalization and quantification of CB1R intensity in the cerebellar molecular layer at the end of a sub-chronic exposure to MIN or SAL on sub-chronic THC (5 and 20 mg/kg) and sub-chronic VEH treatment conditions (*n*=4 mice per group; 3-4 images per mouse). Note the CB1R down-regulation in the molecular layer of the cerebellum 5 d after the end of the THC-5 and THC-20 sub-chronic administration. ml: molecular layer, PC: Purkinje cell layer, gl: granular layer. Scale bar: 75 µm. ★P<0.05, ★★P<0.01, ★★★P<0.001 vs. sub-chronic VEH+SAL (5 d); ☆P<0.05, ☆☆P<0.01, ☆☆☆P<0.001 vs. sub-chronic THC (5 or 20)+SAL (5 d).

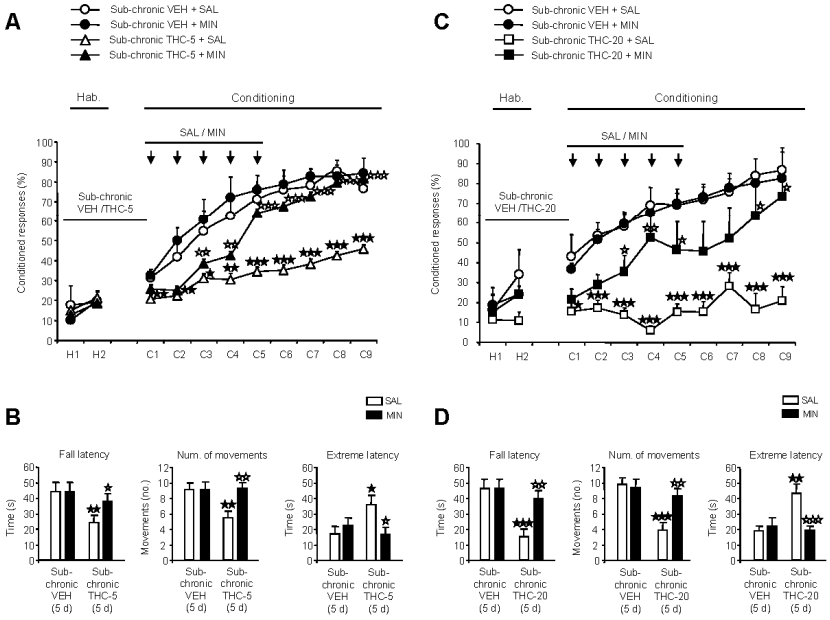

Figure 4. Minocycline treatment prevents the cerebellar deficits produced by THC exposure. **(A)** Percentage of conditioned eyelid responses collected from mice receiving MIN or SAL after THC-5 or VEH sub-chronic treatment (*n*=9-11 mice per group) (see Supplementary Figure 1B for experimental chronogram). **(B)** Motor coordination analysis at the end of a sub-chronic exposure to MIN or SAL on mice that received previously THC-5 or VEH (*n*=15 mice per group). The alterations in motor coordination evaluated in the coat-hanger test were ameliorated by MIN sub-chronic administration. **(C)** Percentage of conditioned eyelid responses collected from mice receiving MIN or SAL after sub-chronic THC-20 or VEH treatment (*n*=9-11 mice per group) (see Supplementary Figure 1B for experimental chronogram). **(D)** Motor coordination analysis at the end of a sub-chronic exposure to MIN or SAL on mice that received previously THC-20 or VEH (*n*=15 mice per group). The impairment in motor coordination skills measured in the coat-hanger test were prevented by MIN sub-chronic treatment. ★P<0.05, ★★P<0.01, ★★★P<0.001 vs. sub-chronic VEH+SAL (5 d); ☆P<0.05, ☆☆P<0.01, ☆☆☆P<0.001 vs. sub-chronic THC (5 or 20)+SAL (5 d).

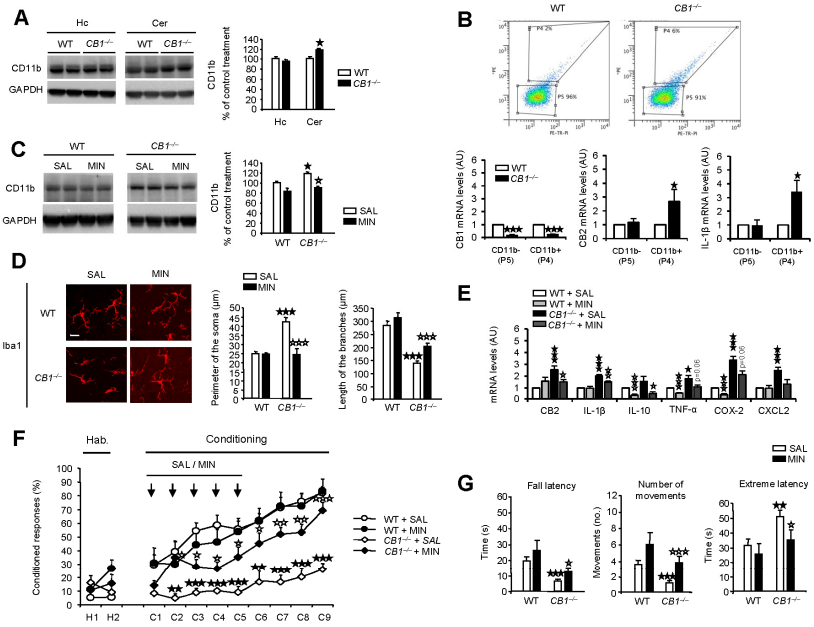

Figure 5. Genetically down-regulated CB1R promotes cerebellar neuroinflammation and reversible deficits in cerebellar function. **(A)** Immunoblot and quantification of CD11b in cerebellar and hippocampal homogenates in WT and CB1R KO (*CB1*$^{-/-}$) mice (*n*=5 per group). CD11b detection was referred to GAPDH. ★P<0.05 vs. WT. **(B)** Flow cytometric analysis for CD11b expression and quantitative RT-PCR analysis for CB1R, CB2R and IL-1β on acutely dissociated cerebellar cells from WT and *CB1*$^{-/-}$ mice (*n*=3-4 per group) sorted on CD11b+ population (P4) and CD11b– population (P5). **(C)** Immunoblot and quantification of CD11b in cerebellar homogenates in WT and *CB1*$^{-/-}$ mice treated with MIN or SAL (*n*=6 mice per group). CD11b detection was referred to GAPDH. ★P<0.05 vs. WT+SAL; ☆P<0.05 vs. *CB1*$^{-/-}$+SAL. **(D)** Morphological analysis on Iba1+ cells in cerebellar cortex (*n*=3-5 mice per group, 5 cells per mouse) of WT and *CB1*$^{-/-}$ mice treated with MIN or SAL. Scale bar: 25 µm. ★★★P<0.001 vs. WT+SAL; ☆☆☆P<0.001 vs. *CB1*$^{-/-}$+SAL. **(E)** mRNA expression analysis by quantitative RT-PCR of CB2R and inflammation-related genes in the cerebellum of WT + SAL, WT + MIN, *CB1*$^{-/-}$ + SAL and *CB1*$^{-/-}$ + MIN mice (*n*=5-6 per group). ★P<0.05, ★★P<0.01, ★★★P<0.001 vs. WT. **(F)** Percentage of conditioned eyelid responses collected from WT and *CB1*$^{-/-}$ mice treated with MIN or SAL (*n*=7-10 mice per group). (see Supplemental Figure 12A for experimental chronogram). ★★P<0.01, ★★★P<0.001, vs. WT + SAL; ☆P<0.05, ☆☆P<0.01, ☆☆☆P<0.001 vs. *CB1*$^{-/-}$ + SAL. **(G)** Motor coordination analysis in WT and *CB1*$^{-/-}$ mice treated for 5 d with MIN or SAL (*n*=13-22 mice per group). ★★P<0.01, ★★★P<0.001, vs. WT+SAL; ☆P<0.05, ☆☆☆P<0.001 vs. *CB1*$^{-/-}$+SAL.

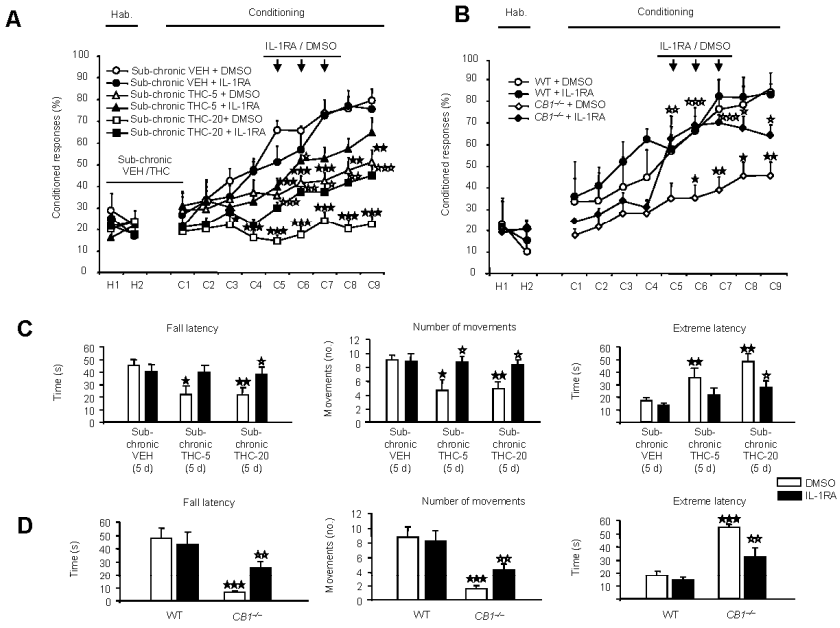

Figure 6. Acute IL-1RA administration resolves the cerebellar deficit resulting from cannabinoid receptor de-regulation and neuroinflammation. **(A)** Percentage of conditioned eyelid responses collected from mice sub-chronically treated with THC (5 and 20 mg/kg) and VEH (n=5-10 mice per group) (see Supplemental Figure 1C for experimental chronogram). ★P<0.05, ★★P<0.01, ★★★P<0.001, vs. sub-chronic VEH+DMSO; ☆P<0.05, ☆☆P<0.01, ☆☆☆P<0.001 vs. sub-chronic THC (5 or 20 mg/kg)+DMSO. **(B)** Percentage of conditioned eyelid responses collected from WT and *CB1*$^{-/-}$ mice (n=5-10 per group) (see Supplemental Figure 12B for experimental chronogram). ★P<0.05, ★★P<0.01 vs. WT+DMSO; ☆P<0.05, ☆☆P<0.01, ☆☆☆P<0.001 vs. *CB1*$^{-/-}$+DMSO. **(C)** Motor coordination analysis in the coat-hanger test in sub-chronic THC (5 and 20 mg/kg) and sub-chronic VEH conditions 5 d after spontaneous withdrawal (n=9-15 mice per group). Four hours before the test, mice received an acute injection of IL-1RA (100 mg/kg, i.p.) or its vehicle (DMSO). ★P<0.05, ★★P<0.01 vs. sub-chronic VEH. **(D)** Motor coordination analysis in the coat-hanger test in WT and *CB1*$^{-/-}$ mice (n=8-13 per group). Four hours before the test, mice received an injection of IL-1RA or its vehicle. ★★★P<0.001 vs. WT+DMSO; ☆☆P<0.01 vs. *CB1*$^{-/-}$+DMSO.

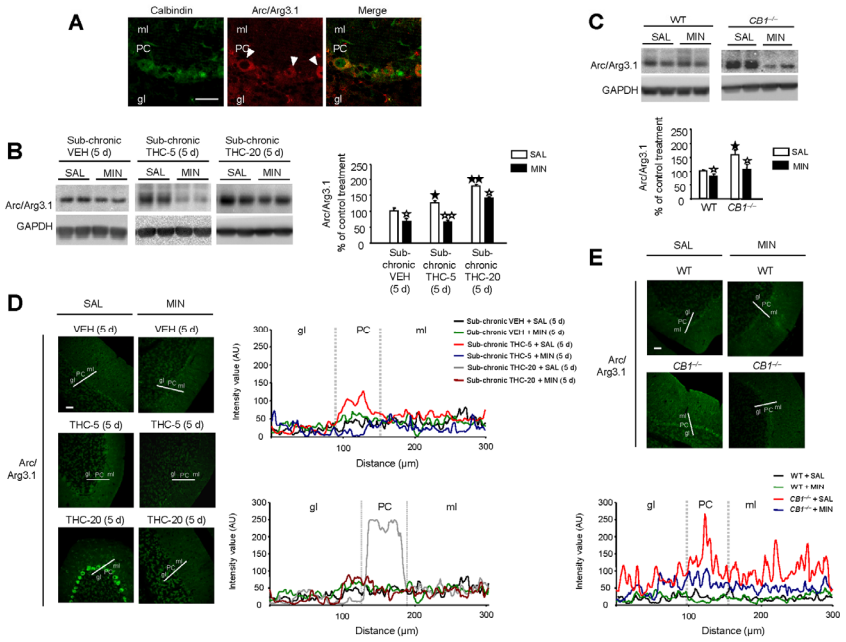

Figure 7. Cerebellar neuroinflammation secondary to cannabinoid down-regulation modulates Arc/Arg3.1 expression. **(A)** Immunofluorescence detection of Arc/Arg3.1 and calbindin in Purkinje neurons in the cerebellar cortex. ml: molecular layer; PC, Purkinje cell layer; gl, granular layer. Scale bar: 50 µm. **(B)** Immunoblot detection and quantification of Arc/Arg3.1 in cerebellar homogenates obtained at the end of a sub-chronic exposure to MIN or SAL on sub-chronic THC (5 and 20 mg/kg) and sub-chronic VEH treatment conditions (n=5-6 mice per group). ★P<0.05, ★★P<0.01 vs. sub-chronic VEH+SAL (5 d); ☆P<0.05, ☆☆P<0.01 vs. sub-chronic THC-5+SAL (5 d) or sub-chronic THC-20+SAL (5 d). **(C)** Immunoblot detection and quantification of Arc/Arg3.1 in cerebellar homogenates from *CB1*$^{-/-}$ and WT mice at the end of a sub-chronic exposure to MIN or SAL (n=5-6 mice per group). ★P<0.05 vs. sub-chronic WT+SAL; ☆P<0.05 vs. WT+SAL. **(D)** Arc/Arg3.1 immunostaining images from mice treated sub-chronically with THC-5, THC-20 or VEH and that received MIN or SAL during 5 d. Arc/Arg3.1 intensity was measured along 300 µm line stretched along the three cerebellar layers. Plot represents Arc/Arg3.1 intensity alongside the layers quantified with the ImageJ software. Scale bar: 100 µm. **(E)** Arc/Arg3.1 immunostaining images from *CB1*$^{-/-}$ and WT mice at the end of a sub-chronic exposure to MIN or SAL. Arc/Arg3.1 intensity was measured along 300 µm line stretched along the three cerebellar layers. Plot represents Arc/Arg3.1 intensity alongside the layers quantified with the ImageJ software. Scale bar: 100 µm.

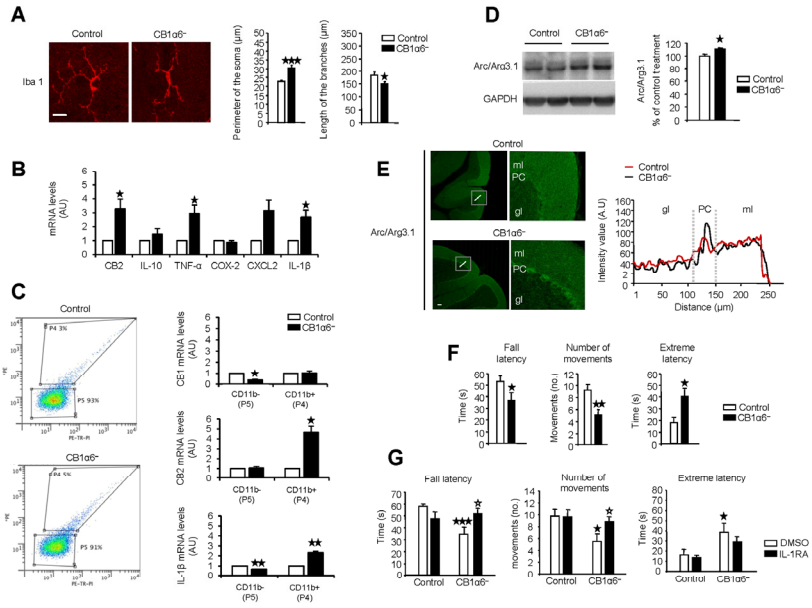

Figure 8. CB1R down-regulation in the parallel fibers is sufficient to trigger cerebellar neuroinflammation and IL1-RA-sensitive motor coordination impairment. **(A)** Morphological analysis on Iba1+ cells in cerebellar cortex of *CB1α6*⁻ and control mice (n=4-5 per group, 6 cells per mouse). Scale bar: 25 µm. ★P<0.05, ★★★P<0.001 vs. control. (**B**) mRNA expression analysis by quantitative RT-PCR of CB2R and inflammation-related genes in the cerebellum of *CB1α6*⁻ and control mice (n=5 per group). ★P<0.05 vs. control. (**C**) Flow cytometric analysis for CD11b expression and quantitative RT-PCR analysis for CB1R, CB2R and IL-1β on acutely dissociated cerebellar cells from *CB1α6*⁻ and control mice (n=3-4 per group) sorted on CD11b+ population (P4) and CD11b– population (P5). ★P<0.05 vs. control. **(D)** Immunoblot detection and quantification of Arc/Arg3.1 in cerebellar homogenates from *CB1α6*⁻ and control (n=5-6 mice per group). ★P<0.05 vs. control. **(E)** Arc/Arg3.1 immunostaining on *CB1α6*⁻ and control mice. Arc/Arg3.1 intensity was measured along 250 µm stretched along the three cerebellar layers. ml: molecular layer; PC, Purkinje cell layer; gl, granular layer. Plot represents Arc/Arg3.1 intensity along the cerebellar layers. Scale bar: 100 µm. **(F)** Motor coordination analysis in *CB1α6*⁻ and control mice (n=11-15 per group). **(G)** Motor coordination analysis in the coat-hanger test in *CB1α6*⁻ and control mice (n=8-10 per group). Four hours before the test, mice received an injection of IL-1RA (100 mg/kg, i.p.) or its vehicle (DMSO). ★P<0.05, ★★P<0.01, ★★★P<0.001 vs. Control. ☆P<0.05 vs *CB1α6* +DMSO.

Mechanisms underlying the cerebellar deficits produced by repeated cannabis exposure

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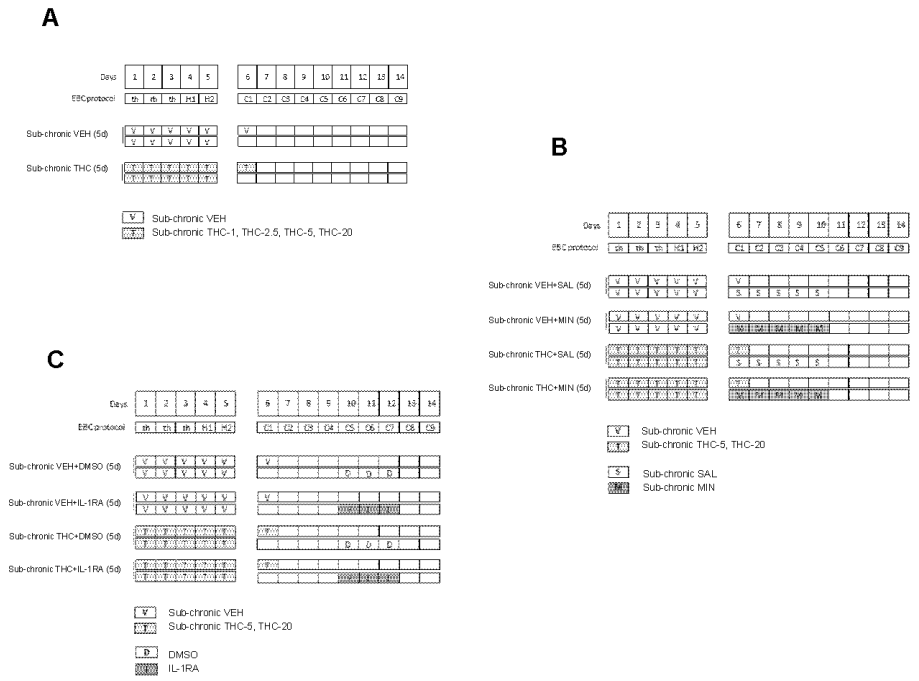
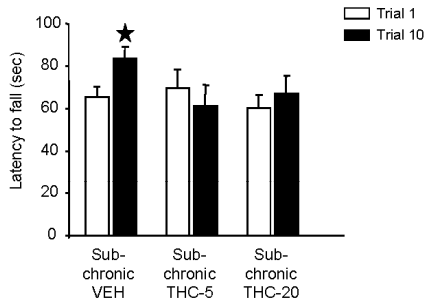
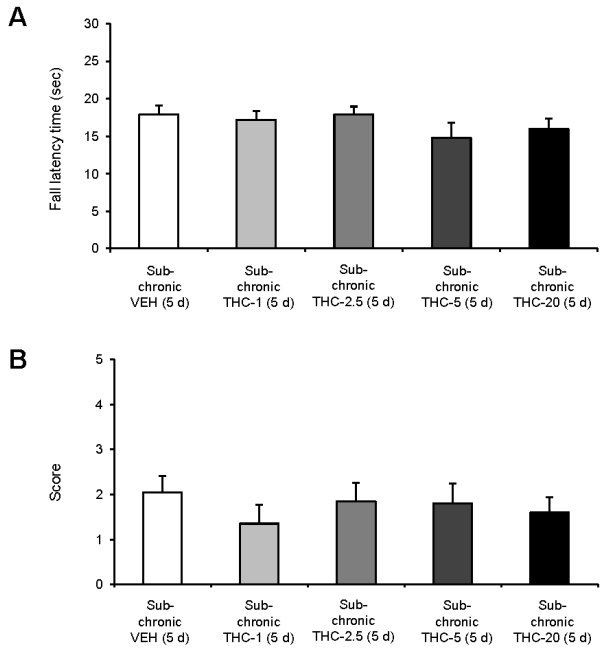


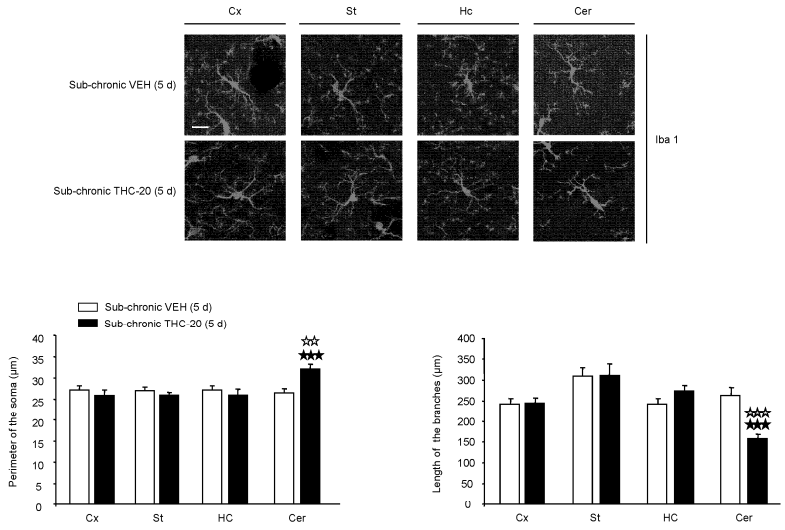
Supplemental Figure 1. Eyeblink conditioning schedules. (**A**) Chronogram for the experiment in Figure 1A. (**B**) Chronogram for experiment in Figure 4, A and C. (**C**) Chronogram for experiment in Figure 6A. Abbreviations: th, Search for thresholds; H, Habituation phase; C, Conditioning phase; T, THC; V, vehicle (VEH), M, minocycline (MIN); S, saline (SAL); I, IL-1RA; D, DMSO.



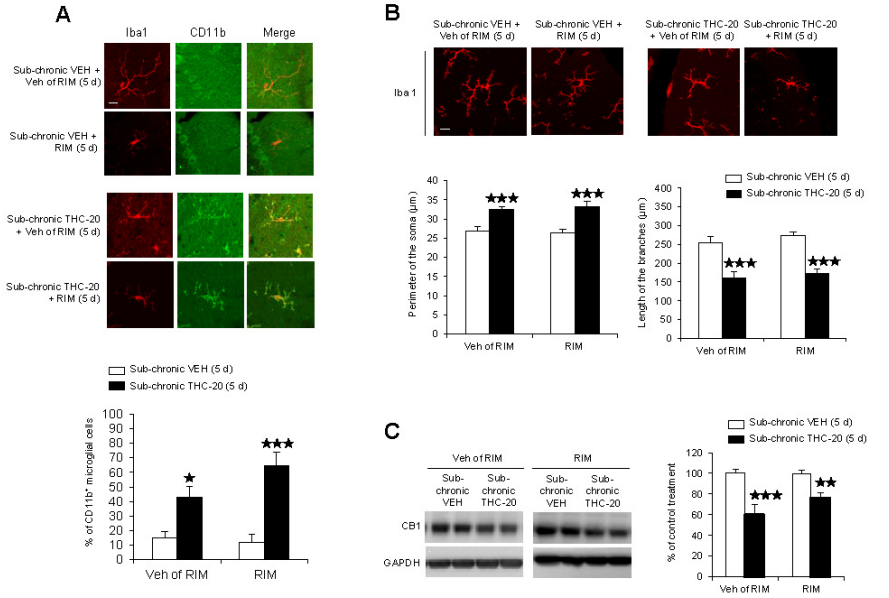
Supplemental Figure 2. Motor coordination performance in the accelerating rotarod after sub-chronic THC exposure. Mice were tested 5 d after the end of treatments with vehicle (sub-chronic VEH), THC (5 mg/kg) (sub-chronic THC-5) and THC (20 mg/kg) (sub-chronic THC-20). The latency to fall in the first trial (Trial 1) and on the tenth trial (Trial 10) of the accelerating rotarod is represented. Mice exposed to THC (n=8-10 per group) showed a similar latency to fall in the first and tenth trial of the test session, while VEH-treated mice did improve in their performance. Data were expressed as mean ± SEM. ★$p<0.05$ vs. first trial.



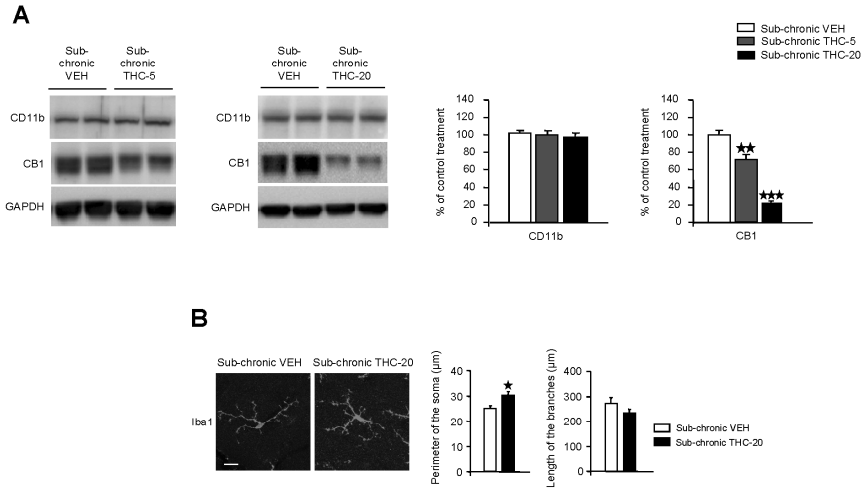
Supplemental Figure 3. Assessment of the equilibrium performance of mice 5 d after THC treatment cessation in the rod test. Mice (n=10 per group) treated with VEH or THC (1, 2.5, 5, 20 mg/kg, i.p.) were tested in the rod test 5 d after the last VEH or THC administration. Results are displayed as fall latency (**A**) or score (**B**). Data were expressed as mean ± SEM.



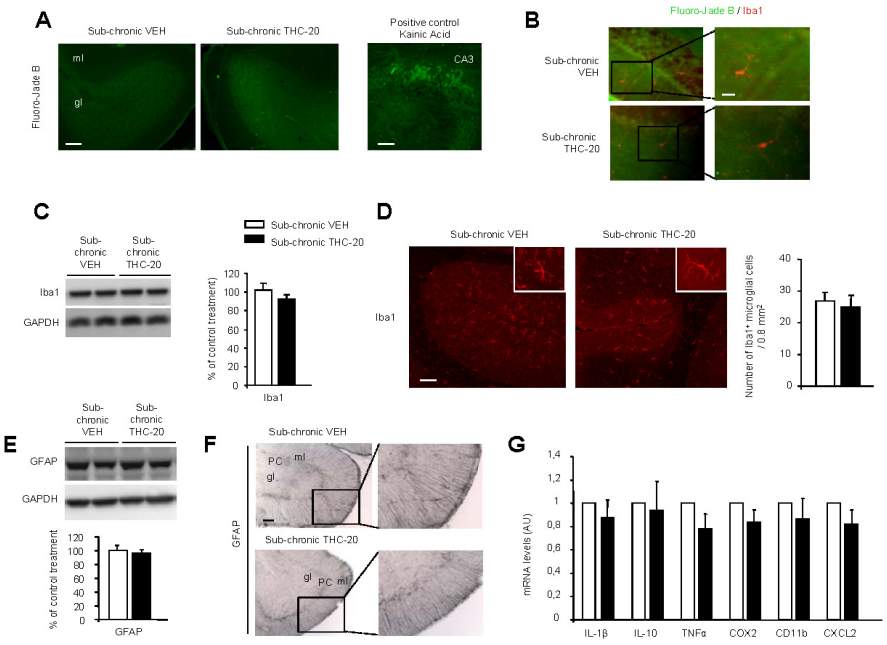
Supplemental Figure 4. Microglial activation 5 d after sub-chronic THC treatment cessation occurs mainly in the cerebellum. Morphological analysis of Iba1+ microglial cells in cortex, striatum, hippocampus and cerebellum from mice treated sub-chronically with VEH or THC-20 and sacrificed 5 d after the last VEH or THC administration (sub-chronic VEH (5 d) or sub-chronic THC-20 (5 d) groups) (n=3-4 mice per group, 5 cells per mouse). The perimeter of the cell soma and the length of the microglial branches from Iba1-stained cells were analyzed with the ImageJ software (see Supplemental Figure 17). Scale bar: 25 µm.



Supplemental Figure 5. The precipitated THC withdrawal similarly promotes the activation of microglial cells detected 5 d after the end of the sub-chronic THC treatment (20 mg/kg, i.p.). (**A**) Immunodetection of Iba1 (red) and CD11b (green) proteins in cerebellar sagittal sections from mice treated sub-chronically with VEH or THC-20 and receiving acutely rimonabant (RIM, 10 mg/kg, i.p.) or its vehicle (n=5 mice per group). The percentage of Iba1+/CD11b+ microglial cells was computed from 25 images (5 images per animal). Scale bar: 25 µm. (**B**) Morphological analysis of Iba1+ microglial cells in cerebellar cortex from mice treated sub-chronically with VEH + Veh of RIM (sub-chronic VEH + Veh of RIM (5 d) group), VEH + RIM (sub-chronic VEH + RIM (5 d) group), THC-20 + Veh of RIM (sub-chronic THC-20 + Veh of RIM (5 d) group) and THC-20 + RIM (sub-chronic THC-20 + RIM (5 d) group) (n=3-5 mice per group, 5 cells per mouse). The perimeter of the cell soma and the length of the microglial branches from Iba1-stained cells were analyzed with the ImageJ software (Supplemental Figure 17). Scale bar: 25 µm. (**C**) Immunoblot and quantification of CB1R in cerebellar homogenates from mice analyzed 5 d after receiving sub-chronically THC-20 or VEH and acutely RIM or its vehicle (n=5 mice per group). The optical density for CB1R detection was referred to the detection of the housekeeping control GAPDH in the same samples. Data were compared between groups (sub-chronic THC-20 + Veh of RIM (5 d) vs. sub-chronic VEH + Veh of RIM (5 d), and sub-chronic THC-20 + RIM (5 d) vs. sub-chronic VEH + RIM (5 d)) using one-way ANOVA and were expressed as mean ± SEM. ★p<0.05, ★★p<0.01, ★★★p<0.001.

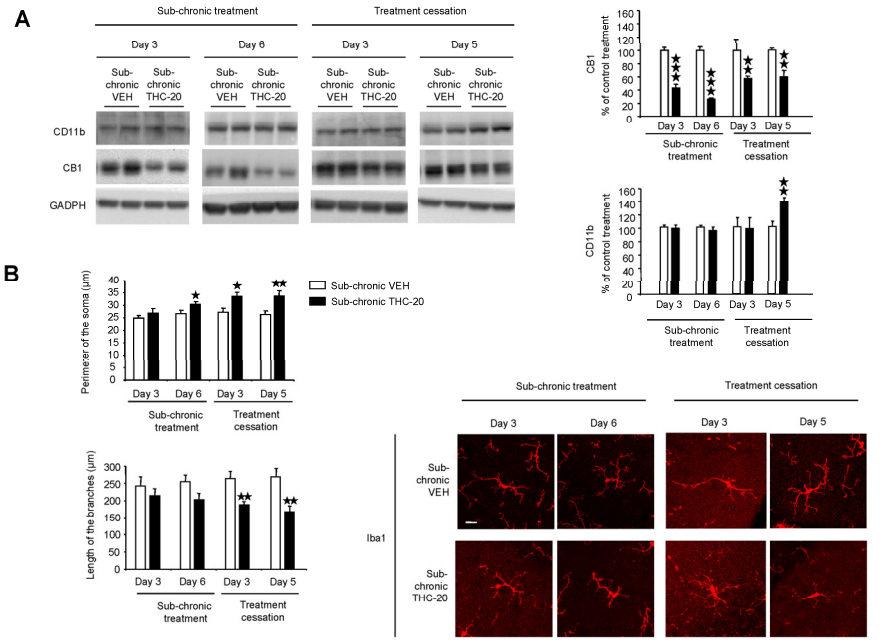


Supplemental Figure 6. Sub-chronic THC administration did not produce CD11b enhancement or a significant change in the microglial morphology. (**A**) Immunoblot and quantification of CD11b and CB1R in cerebellar homogenates from mice processed at the end of THC (5 or 20 mg/kg, i.p.) or VEH sub-chronic treatment (n=5 mice per group). The optical density for CD11b and CB1R detection was referred to the detection of the housekeeping control GAPDH in the same samples. (**B**) Morphological analysis of Iba1+ microglial cells in the molecular layer of the cerebellum from mice treated sub-chronically with VEH or THC 20 mg/kg and sacrificed after 5 d (n=5 mice per group, 5 cells per mouse). The perimeter of the cell soma and the length of the microglial branches from Iba1-stained cells were analyzed with the ImageJ software (Supplemental Figure 17). Scale bar: 25 µm. Data were compared between groups using one-way ANOVA and expressed as mean ± SEM. ★p<0.05, ★★p<0.01, ★★★p<0.001.

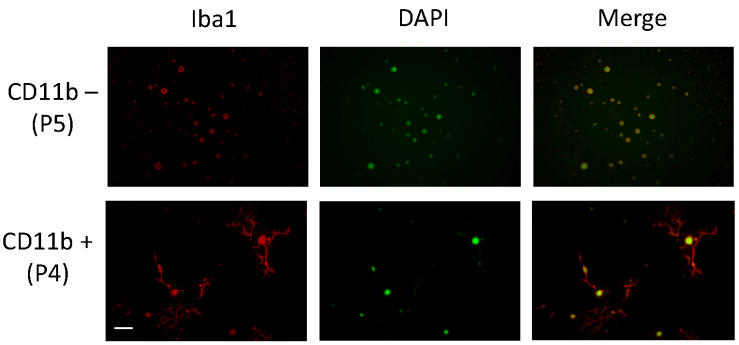


Supplemental Figure 7. No signs of cellular stress neither microglial or astroglial proliferation were detected at the end of the THC-20 sub-chronic treatment. (**A**) Brains were removed 30 min after the last THC-20 or VEH administration and processed for Fluoro-Jade B assay (n=4 mice per group). As a positive control, an independent group of mice (n=3 per group) was treated with an excitotoxic dose of kainic acid (15 mg/kg, i.p.) and their brains were processed for Fluoro-Jade B staining 3 d later. There was no sign of cellular death in the cerebellar cortex after sub-chronic THC-20 administration. As expected, the CA3 region of the hippocampus in the positive control group showed Fluoro-Jade B staining corresponding to cellular death. Scale bars: 100 µm (cerebellum), 50 µm (hippocampus). (**B**) Representative double labeling for Iba1 (red) and Fluoro-Jade B (green) in sagittal cerebellar sections from sub-chronic VEH and sub-chronic THC-20 mice (n=3 per group). Fluoro-Jade B reaction did not produce specific staining in the cerebellum of THC-20 treated mice. Scale bar: 25 µm. (**C**) Immunoblot detection of Iba1 in cerebellar homogenates from mice exposed to sub-chronic THC-20 or VEH (n=5 mice per group). Optical density analysis of Iba1 was referred to GAPDH detection in the same samples. (**D**) Immunolocalization of Iba1 in the cerebellar cortex of mice at the end of the sub-chronic THC-20 or VEH treatment. See inset for representative microglial morphological change. Iba1+ cells were counted in images representing an area of 0.8 mm^2 per optical field (n=4 mice per group, 5 images per mouse). Scale bar: 100 µm. (**E**) Immunoblot detection of glial fibrillary acidic protein (GFAP) in cerebellar homogenates from mice exposed to sub-chronic THC-20 or VEH (n=4 per group). Optical density analysis of GFAP was referred to GAPDH detection in the same samples. (**F**) Immunohistochemical detection of GFAP after sub-chronic THC-20 or VEH exposure. Bright field images of stained sagittal cerebellar sections are presented in gray-scale for comparative purposes. All brain samples were collected 30 min after the last THC-20 or VEH administration (n=3 mice per group). Scale bar: 100 µm. (**G**) mRNA expression analysis by real time quantitative PCR of pro-inflammatory factors at the end of the sub-chronic THC-20 or VEH treatment (n=7-8 per

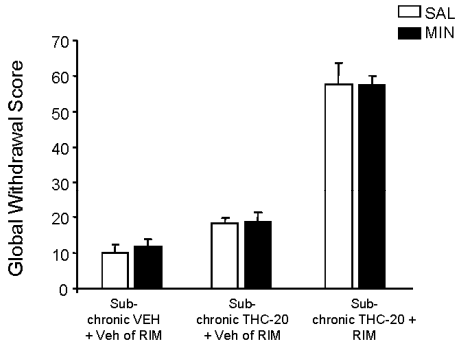
group). Cytokines mRNA levels were referred to GAPDH mRNA detection in the same samples. Abbreviations: ml: molecular layer; PC: Purkinje cell layer; gl: granular layer; CA3, CA3 field of Ammon's horn. Data were analyzed using one-way ANOVA and expressed as mean ± SEM.



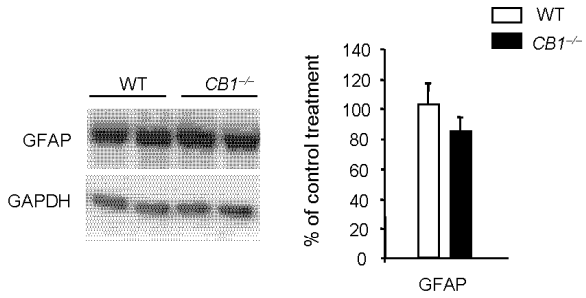
Supplemental Figure 8. Time course for the expression of CD11b, CB1R and the alteration in cerebellar microglia morphology during pharmacological treatment and after THC withdrawal. (**A**) Immunodetection and quantification for CD11b, CB1R and GAPDH on the same samples of cerebellar homogenates. Cerebellar tissues were obtained at different time points during the sub-chronic THC treatment (Day 3), at the end of the sub-chronic THC treatment (Day 6), 3 d after the end of sub-chronic THC treatment, and 5 d after the end of sub-chronic treatment. CD11b and CB1R expression was corrected by the detection of GAPDH. (**B**) Microglial morphology was analyzed on day 3 and 6 during sub-chronic treatment, and at day 3 and 5 after THC cessation. Confocal images were acquired and analyzed with the ImageJ software (Supplemental Figure 17). Scale bar: 25 µm. Data were expressed as mean ± SEM. ★p<0.05, ★★p<0.01, ★★★p<0.001 vs. sub-chronic VEH.



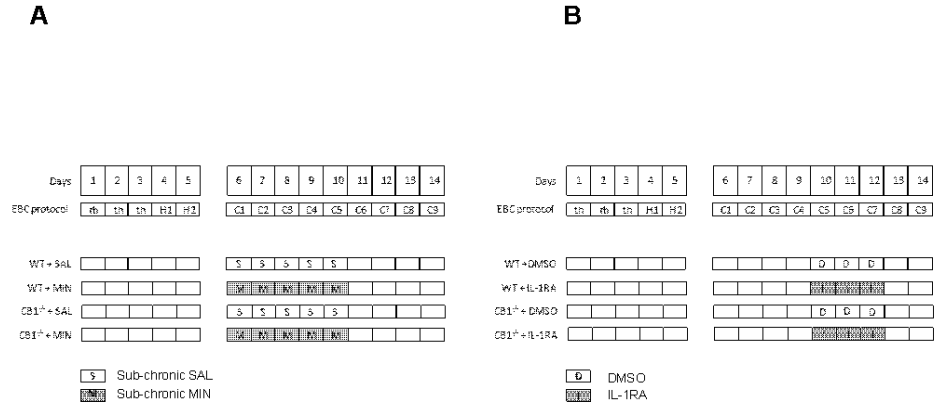
Supplemental Figure 9. CD11b+ (P4) population is highly enriched in Iba1+ cells. Staining for Iba1 and DAPI in CD11b+ (P4) and CD11b– (P5) cells sorted after acute dissociation of cerebellar tissue. Note that Iba1 staining appeared in CD11b+/DAPI+ cells, while it was not observed in CD11b–/DAPI+ cells. Scale bar: 25 µm.



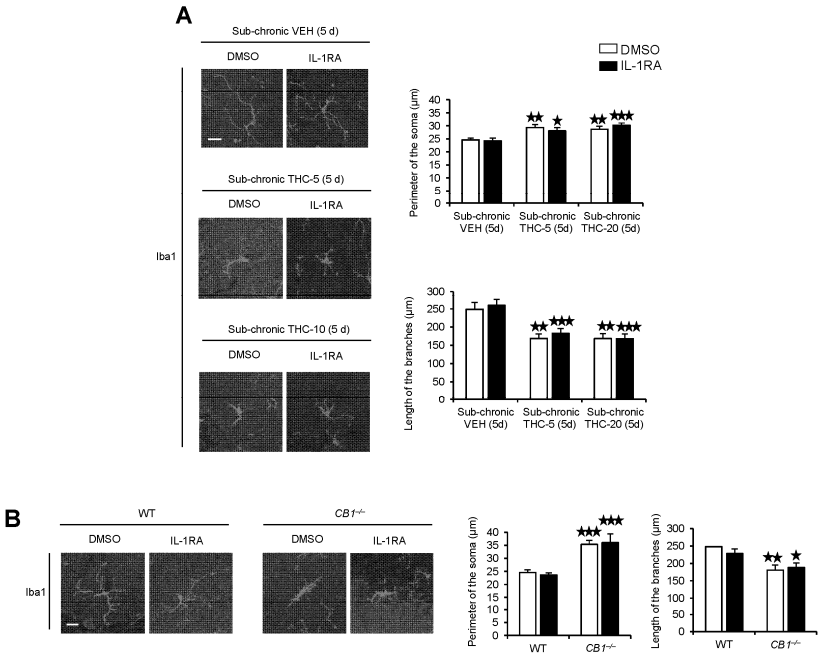
Supplemental Figure 10. Precipitated cannabinoid withdrawal syndrome is not affected by acute minocycline administration. Three hours after the last sub-chronic administration of THC (20 mg/kg, i.p.) or vehicle, mice received an administration of minocycline (MIN, 40 mg/kg, i.p.) or saline (SAL), and 1 hour later they were injected with rimonabant (RIM, 10 mg/kg, i.p.) or its vehicle (VEH) (n=10 mice per group). Then, mice were observed during 45 min and the somatic signs of withdrawal (paw tremor, wet dog shakes, sniffing, writhing, jumping, ptosis, piloerection, ataxia, mastication, locomotor activity, hunched posture and penil lick) were quantified. A global withdrawal score was obtained as described in the Methods section. RIM precipitated the characteristic cannabinoid withdrawal syndrome in mice treated sub-chronically with THC-20 and MIN administration did not alter the behavioral manifestations of cannabinoid withdrawal. Data were expressed as mean ± SEM.



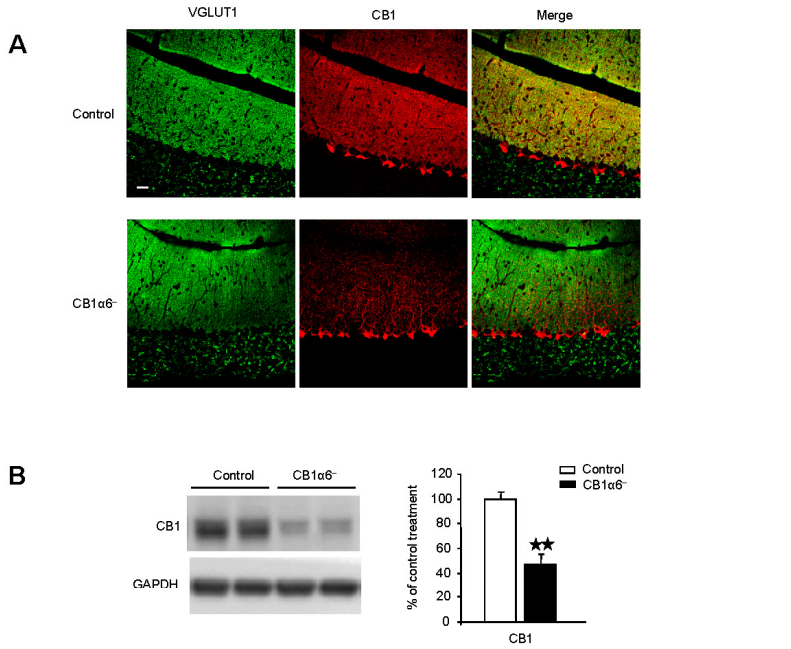
Supplemental Figure 11. Representative immunoblot and quantification of cerebellar GFAP in $CB1^{-/-}$ line (n=6 mice per group). No differences were observed in GFAP expression. Data were expressed as mean ± SEM.



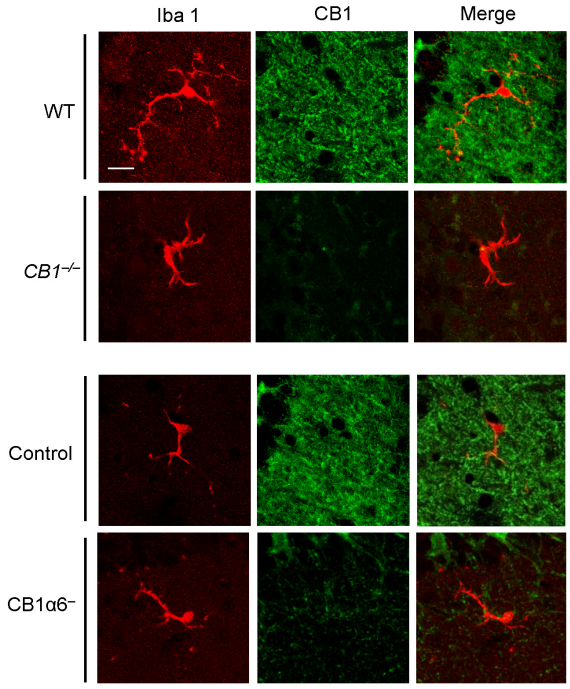
Supplemental Figure 12. Eyeblink conditioning schedules. (**A**) Chronogram for the experiments in Figure 5F. (**B**) Chronogram for the experiments in Figure 6B. Abbreviations: th, Search for thresholds; H, Habituation phase; C, Conditioning phase; T, THC; V, vehicle (VEH); M, minocycline (MIN); S, saline; I, IL-1RA; D, DMSO.



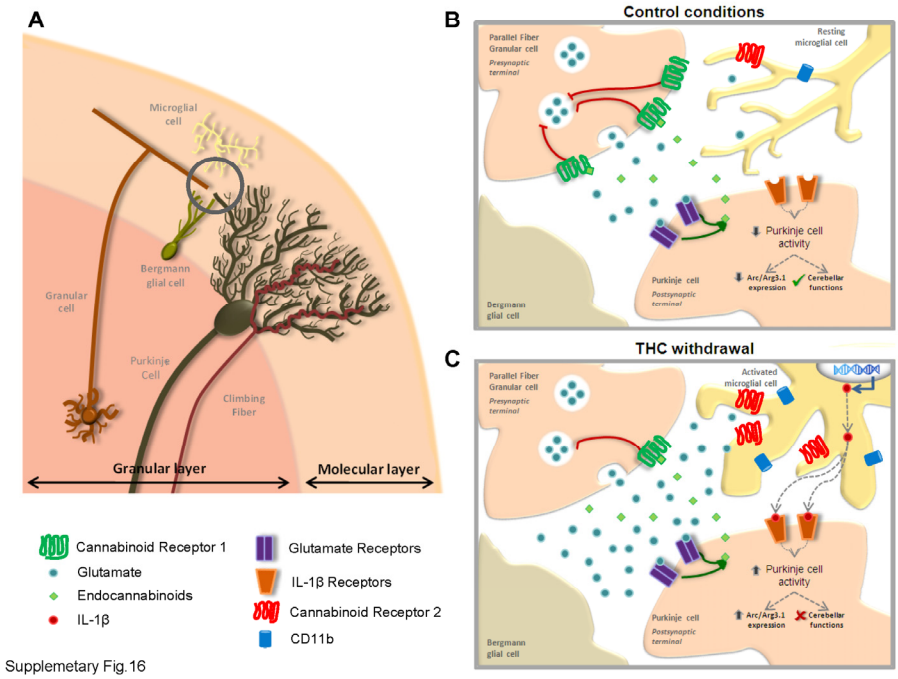
Supplemental Figure 13. IL-1RA acute administration does not modify microglial morphology. (**A**) The perimeter of the cell soma and the length of the microglial branches from Iba1-stained cells were analyzed in THC-withdrawn mice and VEH controls treated acutely with IL-1RA (100 mg/kg, i.p., 4h) or its vehicle (DMSO). Cerebellar sections were stained with Iba1 to reveal microglial morphology. Confocal images were analyzed with the ImageJ software as indicated in Supplemental Figure 17. Scale bar: 25 µm. Data were expressed as mean ± SEM. ★p<0.05, ★★p<0.01, ★★★p<0.001 vs. sub-chronic VEH. (**B**) Analysis of the cerebellar microglial phenotype from $CB1^{-/-}$ and WT mice after acute administration of IL-1RA (100 mg/kg, i.p., 4 h). Cerebellar sections were stained with Iba1 to reveal microglial morphology. Confocal images were analyzed with the ImageJ software as indicated in Supplemental Figure 17. Scale bar: 25 µm. Data were expressed as mean ± SEM. ★p<0.05, ★★p<0.01, ★★★p<0.001 vs. WT+DMSO.



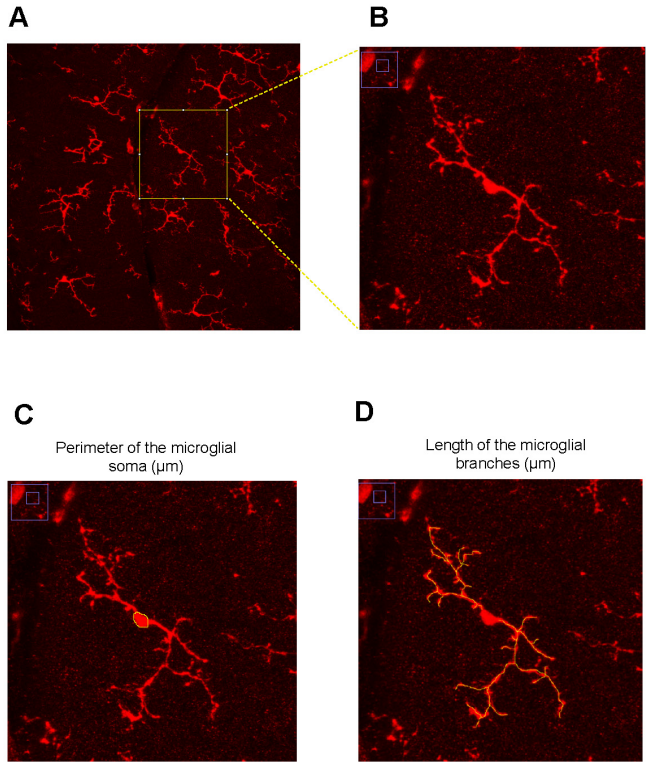
Supplemental Figure 14. Localization and expression of CB1R in the cerebellar cortex of CB1α6⁻ line. (**A**) Immunofluorescence for VGLUT1 and CB1R in control and CB1α6⁻ mice. Note the specific removal of CB1R in the parallel fibers on the molecular layer of the cerebellum in CB1α6⁻ mice, without affecting VGLUT1 expression. Scale bar: 75 μm. (**B**) Immunoblot detection and quantification of CB1R in cerebellar homogenates of control and CB1α6⁻ mice (n=4 mice per group). Data expressed as mean ± SEM. ★★$p<0.01$ vs. control.



Supplemental Figure 15. Expression of low to undetectable levels of CB1R in microglial cells. Sections from WT, $CB1^{-/-}$, control and $CB1\alpha6^{-}$ mice were stained in parallel for CB1R and Iba1. CB1R signal was low to undetectable and did not change under neuroinflammatory conditions in Iba1+ cells on $CB1\alpha6^{-}$ mice. $CB1^{-/-}$ sections were used to determine the background signal of the assay. Scale bar: 25 µm.



Supplemental Figure 16. Proposed model for the changes observed after sub-chronic THC exposure in the molecular layer of the cerebellum. (**A**) General organization of the main cellular components in the cerebellar cortex discussed in this study. (**B**) Under control conditions, CB1R located in the presynaptic parallel fibers modulate glutamatergic release in the Purkinje cell dendrites through highly ensheathed synapses. Quiescent microglial cells survey the synaptic and extrasynaptic gap. (**C**) After sub-chronic THC treatment –or genetic dysruption of CB1R–, the CB1R down-regulation would result in the localized perturbation of glutamate handling. This glutamate de-regulation could enhance the activity of glutamate receptors expressed by microglial cells, promoting the classical activation characterized by morphological changes and enhancement in the CB2R and IL-1β transcription. IL-1β acting on the IL-1 receptors expressed in the Purkinje cell dendrites, could increase the Purkinje cell activity, as it was revealed by changes in Arc/Arg 3.1 expression. Activated microglia, alters cerebellar associative learning after sub-chronic THC exposure, and both, minocycline and IL-1R antagonist treatment, restore cerebellar function by reducing microglial activation and IL-1β action into the Purkinje cell, the main output of the cerebellar cortex.



Supplemental Figure 17. Methodology to measure microglial cell morphology in brain slices. (**A**) Images from Iba1 stained cells were acquired as Z-stacks (section of 15µm of with), to analyze the whole cell architecture. (**B**) Flattened images were examined by creating a region of interest (ROI) that would include the cell of interest entirely. The perimeter of the soma (**C**) and the length of the microglial branches (**D**) were measured using the ImageJ software tool "Freehand line", as depicted. Afterwards, both parameters were recorded using the ImageJ software option "Analyze and Measure".

MoU and PATENTS

Inventors: A. Busquets, A. Ozaita, R. Maldonado

Title: Dispositivo para la realización de pruebas cognitivas en animales

Application number:U200802592

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Inventors: A. Ozaita, E. Puighermanal, A. Busquets, R. Maldonado

Title: Temsirolimus for use in the prevention and/or treatment of a delta 9-tetrahydrocannabinol-induced anxiety disorder

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Holding entity: UNIVERSITAT POMPEU FABRA

Countries of extension: -

Exploiting company: n.a.

"Sólo si me siento valioso por ser como soy, puedo aceptarme, puedo ser auténtico, puedo ser verdadero"

Jorge Bucay

"Las personas que piensan que no son capaces de hacer algo, no lo harán nunca, aunque tengan las aptitudes"

Indira Gandhi